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# STUDIES OF INTERMEDIARY METABOLISM IN THE COTYLEDONS OF GERMINATING PEAS, WITH REFERENCE TO THE ROLE OF THE TRICARBOXYLIC ACID CYCLE

by

DONALD STEWART CAMERON

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled STUDIES OF INTERMEDIARY METABOLISM IN THE COTYLEDONS OF GERMINATING PEAS, WITH REFERENCE TO THE ROLE OF THE TRICARBOXYLIC ACID CYCLE submitted by Donald Stewart Cameron in partial fulfilment of the requirements for the degree of Master of Science.

Date ....................

# ABSTRACT

The low rate of $CO_2$ evolution from certain $C^{14}$-labelled substrates has led to the suggestion that the tricarboxylic acid cycle might not be the major respiratory pathway in germinating seeds. The present investigations were designed to determine the extent to which the cycle is operating in the cotyledons of germinating peas. This was examined by supplying products of glycolysis known to occur naturally, ethanol and lactate, labelled with $C^{14}$. Reaction sequences were confirmed by supplying in addition, acetaldehyde-$C^{14}$, acetate-$C^{14}$ and labelled acids associated with the cycle.

These studies showed the presence of a strikingly active TCA cycle which appears to be the focus of extensive metabolic activity. $C^{14}$ was incorporated rapidly into the acids of the cycle as well as a large number of compounds not participating directly in it. The large pools of glutamate and citrate became heavily labelled, resulting in so significant a dilution of $C^{14}$ that only small amounts were released as $CO_2$.

The presence of enzymes catalyzing some of the principal reactions was established and their specific activities were estimated. In addition, the possible significance of dark fixation of $CO_2$ in relation to the TCA cycle was considered.

# ACKNOWLEDGEMENTS

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| | |
|---|---|
| ADP | Adenosine-5'-Diphosphate |
| AMP | Adenosine-5'-Monophosphate |
| ATP | Adenosine-5'-Triphosphate |
| CoA | Coenzyme A |
| FAD | Flavin Adenine Dinucleotide |
| $NAD^+$ | Oxidized Nicotinamide-adenine Dinucleotide |
| NADH | Reduced Nicotinamide-adenine Dinucleotide |
| $NADP^+$ | Oxidized Nicotinamide-adenine Dinucleotide Phosphate |
| NADPH | Reduced Nicotinamide-adenine Dinucleotide Phosphate |
| PEP | Phospho-enolpyruvate |
| TCA Cycle | Tricarboxylic Acid Cycle |
| TPP | Thiamine Pyrophosphate |

Nomenclature of enzymes is according to the International Union of Biochemistry (1961), *Report of the Commission on Enzymes.*

Temperatures are in °C.

# INTRODUCTION

Germination of seeds is characterized by extensive and diverse metabolic reactions the final result of which is growth of the embryo. These reactions are of three main types: breakdown of materials present in the seed, synthesis of new compounds from these breakdown products, and transport of these substances from the storage organ to the embryo. In general, the only external factors involved are water, oxygen, and carbon dioxide (Mayer and Poljakoff-Mayber 1963). The course of these transformations depends upon the nature of the storage material, the permeability of the seed coat to gases, and the characteristic enzyme systems present or latent within the seed. Energy for these reactions is provided by oxidation of the storage product, be it lipid material, which is characteristic of seed tissues; carbohydrate; or infrequently protein (Mayer and Poljakoff-Mayber 1963).

In the present studies, detached cotyledons of germinating seeds of the pea (*Pisum sativum* L.) have been examined to ascertain some of the metabolic pathways associated with the tricarboxylic acid cycle.

## SURVEY OF LITERATURE

### Glycolysis and Fermentation

The chief storage product of the pea seed is carbohydrate, principally starch, which accounts for almost one-half of the air-dry weight (Mayer and Poljakoff-Mayber 1963). As a result of a series of reactions, involving several enzymes, which are not fully elucidated (Whelan 1961), starch is converted to hexose units which are then metabolized by the Embden-Meyerhof-Parnas sequence to pyruvate (Beevers 1961, Conn and Stumpf 1963).

Under conditions where pyruvate production exceeds its oxidative removal, typical anaerobic products are formed (Beevers 1953). These conditions occur naturally in fruits, root tips, and germinating seeds (James 1953) and can be experimentally imposed by selective inhibition of enzymatic pyruvate oxidation, acceleration of pyruvate production, or exclusion of $O_2$ (Beevers 1961). Many if not all seeds experience such a period of natural anaerobiosis during the earliest stages of germination because the intact testa admits insufficient $O_2$. Under these circumstances, pyruvate oxidation is suppressed, and ethanol and lactic acid may accumulate (James 1953). Ethanol is the more usual product in plant tissues as a result of irreversible decarboxylation of pyruvate and subsequent reduction of acetaldehyde, although lactate is produced in some plants by direct reduction of pyruvate (Davies 1959, Cossins 1964).

The Embden-Meyerhof-Parnas sequence has been demonstrated

in a partially purified extract from pea seeds with the addition of ATP, NAD$^+$, and Mg$^{++}$ (Hatch and Turner 1958). The extract catalyzed the conversion of starch, hexoses, glucose -1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate to $CO_2$ and ethanol in a ratio of 1:1 and regenerated ATP and NAD$^+$. The results indicated that alternative glycolytic pathways are not operating to a significant extent.

## Formation of Acetyl Coenzyme A

Pyruvate produced under normal aerobic conditions is converted to acetyl CoA by the pyruvate oxidation enzyme complex, pyruvate:lipoate oxidoreductase (acceptor acylating) 1.2.4.1, acetyl CoA:dihydrolipoate S-acetyl transferase 2.3.1.12, and NADH:lipoamide oxidoreductase 1.6.4.3. The reaction, an oxidative decarboxylation, proceeds in 4 stages and requires Mg$^{++}$, TPP (Figure 2), lipoate, and NAD$^+$, in addition to CoA (Conn and Stumpf 1963). Lactate is presumably reoxidized to pyruvate in order to undergo the same reaction, although in peas much of it is converted to alanine (Cossins 1964).

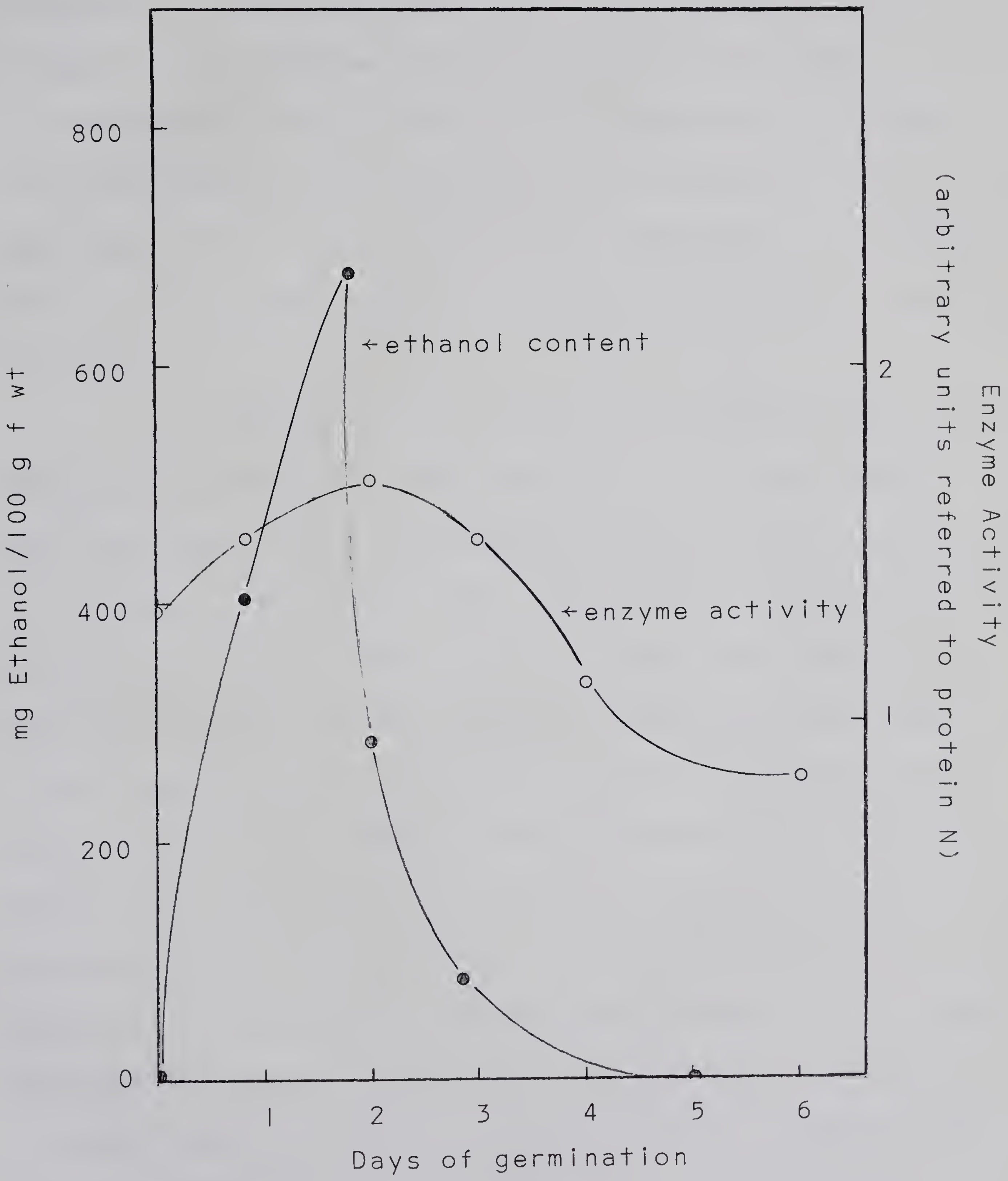
Ethanol, however, cannot readily regenerate pyruvate. Indeed the occurrence of ethanol metabolism in plants was in dispute as cited by Cossins (1961) and Peterson (1964) until confirmed by recent studies (Cossins and Turner 1959). A wide range of plant tissues has since been shown to metabolize ethanol (Cossins and Beevers 1963). In pea seeds the rate of utilization increases if the testa has been ruptured either arti-

ficially by making an incision, or naturally by growth of the radicle. Complete removal of the testa induces further acceleration (Cossins and Turner 1963). Under normal germination conditions both the amount of accumulated ethanol (Cossins 1961) and the activity of alcohol:NAD$^+$ oxidoreductase 1.1.1.1 (Goksöyr *et al* 1953, Maffei Faccioli 1959, Cossins unpublished data) are maximal after approximately 48 hours. At this time the radicles are emerging, resulting in abrupt loss of ethanol and more gradual decrease of enzyme activity (Figure 1). The enzyme is of widespread occurrence in plants, especially in seeds (Cossins and Turner 1962).

Ethanol is converted to acetyl CoA via acetaldehyde but the precise mechanism has not been established. Even though alcohol:NAD$^+$ oxidoreductase strongly favors the formation of ethanol (Fruton and Simmonds 1960), the equilibrium mixture contains a small but significant amount of acetaldehyde, which, through constant withdrawl, results in the complete depletion of ethanol. The oxidation of acetaldehyde to acetyl CoA in bacteria has been shown to require no cofactors but CoA and NAD$^+$ (Burton and Stadtman 1953). However an extract of 2-oxo-acid carboxy-lyase 4.1.1.1 from yeast, requiring TPP, incubated with pyruvate-$C^{14}$ for 5 to 10 seconds produced 'active' acetaldehyde-$C^{14}$ ($\alpha$-hydroxyethyl-$C^{14}$ TPP) which reacted with free acetaldehyde to form acetoin-$C^{14}$ (Holzer and Beauchamp 1961, Miller *et al* 1962). The same compound was isolated from reactions between TPP and either pyruvate or acetaldehyde in

FIGURE I

ALCOHOL:NAD$^+$ OXIDOREDUCTASE ACTIVITY
AND ETHANOL CONTENT IN GERMINATING PEAS



*Data from Cossins (1961) and Maffei Faccioli (1959)*

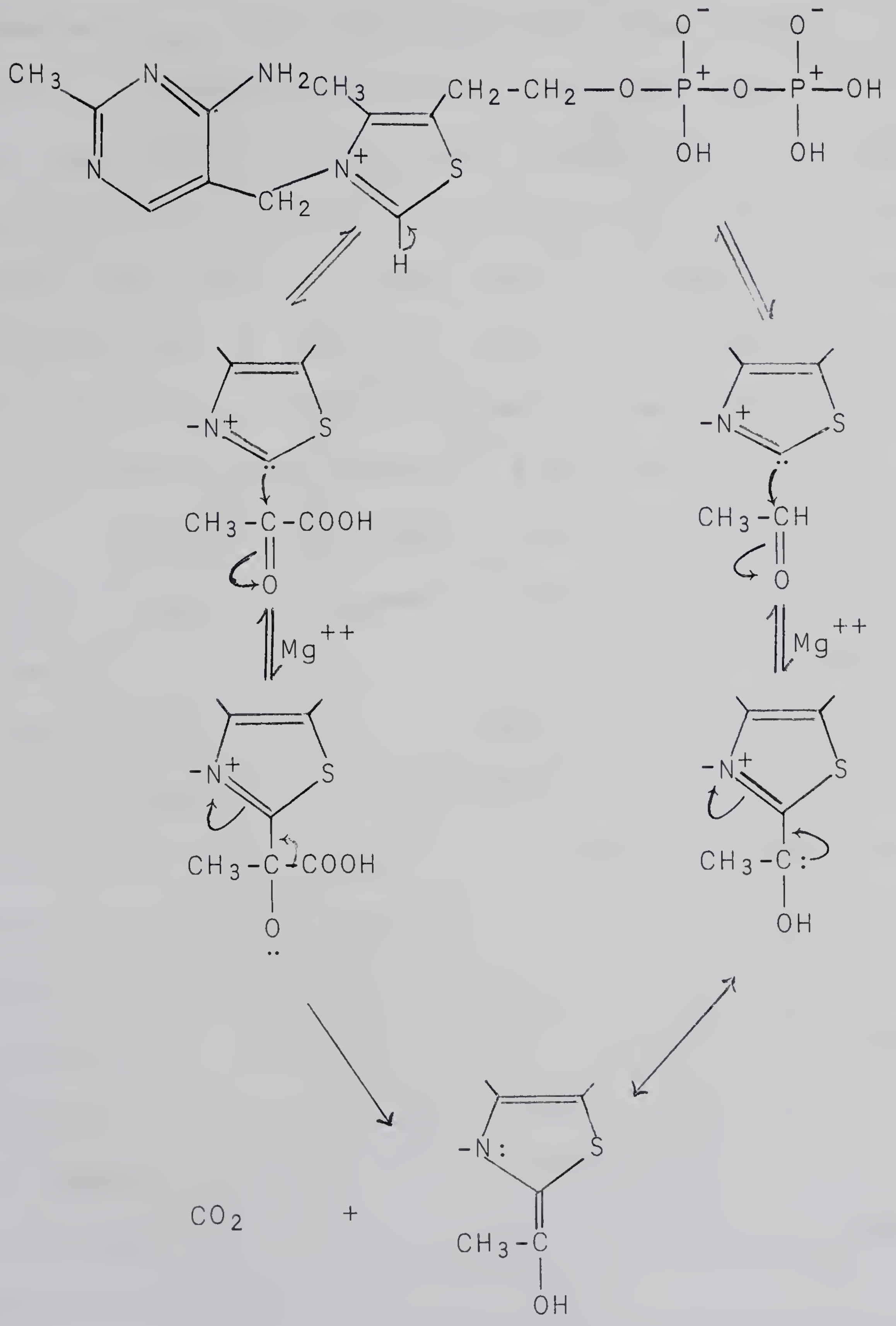
wheat germ (Carlson and Brown 1961). The properties of aldehyde:NAD$^+$ oxidoreductase (acylating CoA) 1.2.1.10 have been examined in cotyledons of germinating peanuts and the presence of the enzyme has been demonstrated in other plant tissues as well (Oppenheim and Castelfranco 1965). Since α-hydroxyethyl TPP and the initial condensation product of acetaldehyde and TPP (Figure 2) are resonance forms of the same compound it seems logical to postulate its further reaction with lipoate, NAD$^+$, and CoA to acetyl CoA in the same manner as pyruvate.

Free acetate is not normally considered as a plant metabolite although it may be present in trace amounts. It has been widely used in metabolic studies and is actively transformed into acetyl CoA. The acetate activating enzyme, acetate:CoA ligase (AMP) 6.2.1.1, was first isolated in plants from an acetone powder of spinach leaves and has been found to be a common constituent of plant tissues (Millerd and Bonner 1954, Hiatt 1962). The mechanism for formation of acetyl CoA from acetate is quite different from the oxidative decarboxylation of pyruvate. Since only Mg$^{++}$ and ATP are required, it presumably involves the formation of an acetyl-AMP-enzyme complex which transfers the acetyl group to CoA, although this intermediate has not been isolated (Fruton and Simmonds 1960).

FIGURE 2

MECHANISMS FOR THE FORMATION OF

α-HYDROXYETHYL THIAMINE PYROPHOSPHATE



*after Breslow (1962) and Conn and Stumpf (1963).*

## The Tricarboxylic Acid Cycle

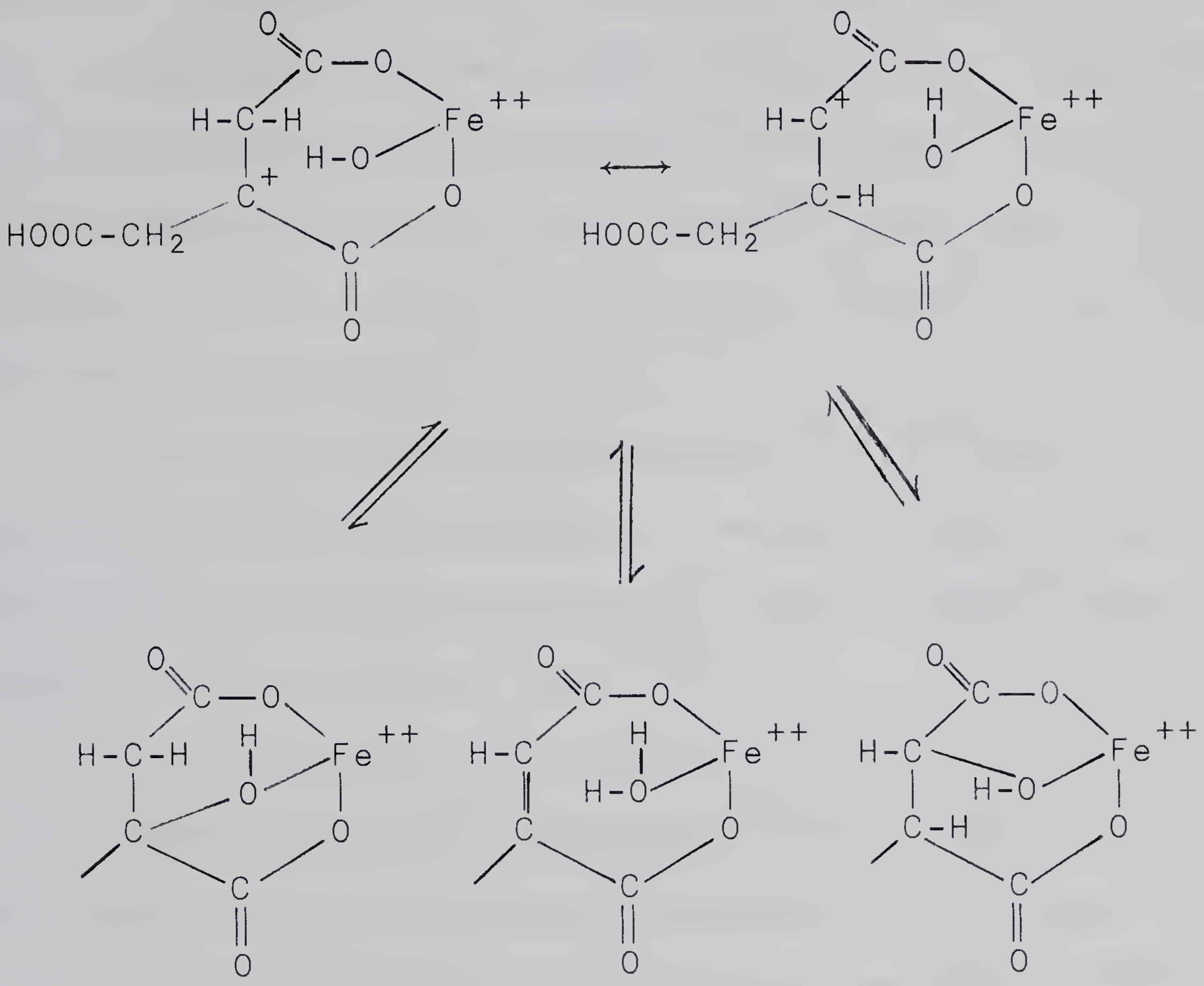
The free energy change of thioester hydrolysis results in a strongly exergonic condensation of acetyl CoA and oxaloacetate; $K_{eq} = 3.2 \times 10^5$ (Conn and Stumpf 1963). The condensing enzyme, citrate oxaloacetate-lyase (CoA acetylating) 4.1.3.7, has been found in many plant tissues and its properties were studied in an acetone preparation from tobacco leaves (Hiatt 1962). The reaction requires no cofactors and none of the intermediates of the cycle cause inhibition. It is interesting to note that even the purified enzyme contains acetate:CoA ligase (AMP) which maintains high activity.

Citrate (isocitrate) hydro-lyase 4.2.1.3, formerly known as aconitase, acts asymmetrically on the symmetrical citrate molecule to form a carbonium ion with $Fe^{++}$ (Speyer and Dickman 1956). This is in keeping with Ogston's deduction that the asymmetrical occurrence of an isotope in a product cannot be taken as conclusive evidence against its arising from a symmetrical precursor. A three point attachment between substrate and enzyme could give rise to sites of attachment of identical groups which would be, however, catalytically different (Ogston 1948). The true intermediate of the reaction is the carbonium ion, in equilibrium with citrate, isocitrate, and cis-aconitate, and interconverted by the enzyme (Figure 3).

Isocitrate is converted to α-ketoglutarate by $D_s$-isocitrate:$NAD^+$ oxidoreductase (decarboxylating) 1.1.1.41

FIGURE 3

THE CARBONIUM ION INTERMEDIATE OF THE REACTION CATALYZED BY CITRATE (ISOCITRATE) HYDRO-LYASE



citrate complex

cis-aconitate complex

isocitrate complex

*after Speyer and Dickman (1956)*

in a mechanism which is not fully elucidated although the enzyme is known to be specific for threo-$D_s$-isocitrate (Vickery 1962). Both $NAD^+$- and $NADP^+$-requiring enzymes have been identified in pea mitochondria (Davies 1955) but the most intensive study of the properties of the enzyme have been made on pig heart preparations (Plaut 1963). The evidence suggests that both the oxidation and decarboxylation reactions are catalyzed by the same enzyme which requires $Mn^{++}$. Oxalosuccinate is apparently bound to the enzyme since little or none of the free acid is present.

The oxidative decarboxylation of α-ketoglutarate is analagous to that of pyruvate. The mechanisms of this and the subsequent reactions from succinyl CoA to oxaloacetate are summarized in Conn and Stumpf (1963).

The presence of an integrated enzyme complex, in the mitochondria, capable of carrying out the reactions of the TCA cycle has been shown in a wide variety of plant tissues. At least in some plants it represents the major pathway of respiration (Hackett 1955). Organic acids also represent intermediates common to the cycle and to concomitant reactions (Hackett 1955, Davies 1959). Studies of acetate-$C^{14}$ utilization have shown that in 30 seconds, 98% of the incorporated radioactivity in the soluble fraction is in organic acids (Harley and Beevers 1963). When pyruvate-$C^{14}$ is supplied, the carboxyl carbon is almost entirely released as $CO_2$ whereas C-2 and C-3 are increasingly incorporated into organic acids

and related compounds (Neal and Beevers 1960). The labelling pattern of these compounds is consistent with the operation of the TCA cycle.

Investigations of oxidative capacities of isolated mitochondria and formation of organic acids show that the TCA cycle appears to be operating during germination (Koller *et al* 1962). All the required enzymes have been found in the mitochondria of pea epicotyls (Davies 1956). In most cases the ability to oxidize the TCA cycle acids changes in parallel with respiration (Hackett 1959).

The cells of higher plants contain relatively large amounts of organic acids: there are theoretical reasons for supposing that a significant proportion of the total organic acid content is present in the vacuole. In studies using acetate-1-$C^{14}$ in a variety of plant tissues, labelling of the TCA cycle acids involved carboxyl carbons exclusively, the carboxyl carbons of malate were equally labelled, and the ratio of labelling of C-5:C-1 of glutamate approached the equilibrium value of 2:1. These patterns are consistent with cycle operation but the specific activities of the acids and $CO_2$ varied greatly. This situation, in which a labelled compound may have a higher specific activity than its precursor, implies that only a fraction of the precursor is involved in the interconversion and that the storage and metabolic pools may only slowly come to equilibrium (MacLennan *et al* 1963).

## Amino Acid Metabolism

The principal means of incorporation of $NH_3$ into amino acids in plants is through the action of L-glutamate:$NAD^+$ oxidoreductase (deaminating) 1.4.1.2 on α-ketoglutarate. Although addition of $NH_3$ to oxaloacetate or pyruvate may stimulate formation of aspartate or alanine, conclusive reports indicating widespread occurrence of enzymes catalyzing such reductive aminations are lacking (McKee 1962). Infiltration of $NH_4^+$ into tissues of plants with different metabolic patterns causes accumulation of glutamate and glutamine as initial products (Kretovich 1965). In addition, L-glutamate: $NAD^+$ oxidoreductase (deaminating) has been isolated from a number of plant sources (Webster 1959, Kretovich 1965). The mechanism of the reaction has not been established but Yakovleva *et al* (1964), using a purified preparation from corn roots, showed that the rate of reductive amination of α-ketoglutarate by $NH_3$ is 8 to 10 times that of oxidative deamination of glutamate. The study also revealed that roots possess higher enzyme activity than leaves.

Ammonia also reacts with glutamate, aspartate, and their substituted derivatives to form amides. The reaction with glutamate, catalyzed by L-glutamate:ammonia ligase (ADP) 6.3.1.2, was studies in peas by Elliott (1953) who found that one of the substrates was activated by ATP. The mechanism of the reaction has recently been extensively studied using a highly purified enzyme from sheep brain (Pamiljans *et al* 1962). First,

glutamate is activated with the simultaneous hydrolysis of ATP to ADP, which proceeds in the absence of $NH_3$. The intermediate remains bound to the enzyme, presumably as glutamyl phosphate. Ammonia displaces the phosphate to form glutamine and releases the enzyme.

The synthesis of asparagine, however, has not been so extensively studied. Various workers have found that plant tissues convert aspartate to asparagine, upon the addition of $NH_3$ and ATP, in a reaction analogous to glutamine formation (Webster 1959). There are no reports as yet of the isolation of this enzyme from higher plant tissues.

Although amides have been regarded as the principal means of reversible storage of $NH_3$, there is evidence to suggest that especially glutamine has a wider importance in general metabolism (Webster 1959).

Transamination is perhaps the most widespread enzymatic activity involving amino acids in plants. It involves reversible transfer of amino groups from amino acids (including those in other than the α-position) and amides to ketoacids (Meister 1955, Webster 1959). Aminotransferase enzymes require pyridoxal-5-phosphate as a cofactor, which forms a Schiff's base between the aldehyde moiety of the pyridoxal and the amino acid (Metzler *et al* 1954).

During germination there is active metabolism of nitrogenous compounds which includes protein hydrolysis, an increase in free amino acids and amides, and protein synthesis

(Webster 1959, Koller *et al* 1962). Recently, there have been several studies of these changes specifically in germinating peas (Goksöyr *et al* 1953, Lawrence *et al* 1959, Lawrence and Grant 1963, Larson and Beevers 1965). The results of these studies show a general decrease of protein, to about half of its original amount in 6 to 8 days, and an initial increase in soluble nitrogenous compounds in the cotyledons as germination proceeds. This suggests hydrolysis of reserve protein to amino acids and amides which are then translocated to the seedling, which shows a marked increase of both soluble and protein nitrogen.

The predominant amino acid of the dry seed is glutamate, with appreciable amounts of asparagine, threonine, glycine, and alanine. After three days of germination the cotyledons still contain a large proportion of glutamate, but it is equalled or surpassed by homoserine. There are also remarkable increases in γ-aminobutyrate, arginine, serine, and glutamine. There is a general increase in the amounts of most amino acids during this period but at a much lower rate. A number of non-protein amino acids have been discovered, including α-aminoadipate (Berg *et al* 1954, Hatanaka and Virtanen 1962), and γ-glutamyl alanine (Virtanen and Berg 1954) in pea cotyledons, and γ-methylene glutamate (and its amide) in another legume, the peanut, *Arachis hypogea* (Done and Fowden 1952). The metabolism of these and other recently isolated amino acids and γ-glutamyl peptides is discussed by

Fowden (1964).

Homoserine was first reported in germinating peas by Virtanen *et al* (1953) and was subsequently discovered to be present in trace amounts in the dry seeds (Berg *et al* 1954). The first step in its synthesis from aspartate in yeast (Black and Wright 1955a,b,c), is the activation of aspartate, requiring ATP and $Mg^{++}$, and is probably analagous to the activation of glutamate in glutamine synthesis. The aspartyl-phosphate complex is reduced by NADPH first to the semialdehyde [L-aspartate β-semialdehyde:$NADP^+$ oxidoreductase (phosphorylating) 1.2.1.11] and then by either NADH or NADPH to homoserine (L-homoserine:$NAD^+$ oxidoreductase 1.1.1.3). The reactions are specific for the L-isomer of aspartate. It is of interest to note that the increase of homoserine in the pea seedling is even greater than in the cotyledons, from which it appears to be translocated. Once formed, homoserine seems to undergo little further metabolism, and is not converted to threonine and methionine of which it is thought to be a precursor (Larson and Beevers 1965).

## Carbon Dioxide Fixation

In the preceeding discussion, no mention of net synthesis as a result of the operation of the pathways has been suggested. Several mechanisms for the fixation of $CO_2$, independent of photosynthetic reactions, are known (Walker 1962), and their physiological significance has been discussed by Davies (1959):

"If an acid, say α-oxoglutarate, is withdrawn from the [TCA] cycle, the cycle will slow down and stop unless an equivalent amount of a $C_4$ acid is produced to combine with acetyl CoA and so maintain the steady state concentration of the cycle acids. Consequently, the amount of carbon dioxide fixed by a cell should be equal to the amount of acid withdrawn from the cycle and proportional to the rate of growth or protein synthesis. It should be pointed out that this argument is valid if other reactions producing $C_4$ acids such as the Thunberg condensation [2 acetyl CoA → succinate] and the condensation of acetyl CoA and glyoxylate, are not quantitatively significant."

$CO_2$ fixation has been shown to occur in a variety of higher plants (Mazelis and Vennesland 1957) and in germinating fatty seeds (Bradbeer 1958, Stiller *et al* 1958). Orthophosphate: oxaloacetate carboxy-lyase (phosphorylating) 4.1.1.31, first discovered in higher plants by Bandurski and Greiner (1953), has a high affinity for its substrates and the free energy of the reaction is large. Since it is virtually irreversible, this system constitutes one of the most effective carboxylating systems known (Bandurski 1955, Walker 1959). The oxaloacetate generated is in the keto form and therefore the P-O bond of the phosphoenolpyruvate must be broken prior to or simultaneously with the $CO_2$ addition (Tchen *et al* 1955).

The present studies were undertaken to assess the role of the TCA cycle and its interrelationship with the other

metabolic reactions occurring in the cotyledons of germinating peas.

# MATERIALS AND METHODS

## Germination Procedure

Seeds of *Pisum sativum* L. cv. 'Homesteader' were grown in moist Vermiculite at 25° for 48 hours after being soaked in distilled water at 25° for 18 hours and then washed in fresh distilled water before planting. At this time the radicle has ruptured the testa and grown to a length of 1.5 to 2.0 cm.

## Time Sequence Experiments

### *Preparation*

After removal of embryos and testas, the cotyledons were sliced with a sharp razor blade to a thickness of approximately 300µ and washed several times in distilled water. The slices were blotted dry on paper towels and for each experiment 0.5 g were placed with 0.5 ml of 0.1M $NaH_2PO_4$ (pH 5.5) in a Warburg flask (capacity 17 ml) with 0.5 ml of 20% (w/v) KOH solution in the centre well to absorb $CO_2$ evolved during the experimental period. Micromolar quantities of radioactive metabolites in 0.1 ml (specific activity 0.3 to 5.8 µc/µmole) were injected uniformly over the slices from a microsyringe. These metabolites, ethanol-1-$C^{14}$, ethanol-2-$C^{14}$, acetaldehyde-1,2-$C^{14}$, acetate-1-$C^{14}$, acetate-2-$C^{14}$, isocitrate-5,6-$C^{14}$, α-ketoglutarate-5-$C^{14}$, glutamate-3,4-$C^{14}$, and lactate-1-$C^{14}$, were supplied by Atomic Energy of Canada Limited, Ottawa; California Corporation for Biochemical Research, Los Angeles;

New England Nuclear Corporation, Boston; and Volk Radiochemical Company, Chicago. The final concentration of these compounds was within the range of 0.9 to 7.4 µmoles/0.6 ml and this volume was such that the slices were maintained in contact with a continuous film of the solution while not totally immersed in it.

The flasks were attached to conventional Warburg manometers at 30° for experimental periods from 5 minutes to 6 hours. In a time sequence of 1 to 5 minutes the procedure was unchanged except that the Warburg flasks and manometers were replaced by Erlenmeyer flasks (capacity 55 ml) in a water bath at 25°, and no $CO_2$ determination was made.

At the end of the experimental period, the contents of the centre well were withdrawn with a microsyringe and added to excess 10% (w/v) $BaCl_2$ solution to precipitate the absorbed $CO_2$ as $BaCO_3$. Metabolic reactions were stopped by addition of absolute ethanol and boiling for 5 minutes to remove volatile substrates.

### *Gross Fractionation*

The fractions separated by the following procedures were assayed for radioactivity using a continuous gas flow detector (Nuclear-Chicago Corporation, Model D47) with a mylar end window. Aliquots of solutions were plated on metal planchets and dried under an infrared lamp in a stream of warm air, after addition of 95% ethanol to ensure even spreading. In all cases counts were corrected for background. Samples

of $BaCO_3$ were also corrected for self-absorption.

The cotyledon slices in ethanol/distilled water solution were transferred to a glass hand grinder and the volume made up to 20 ml with 80% ethanol. After grinding the suspension was centrifuged at 10000 × *g* for 20 minutes and the supernatant solution collected in a flask. The insoluble residue was successively extracted with 20 ml of 50% ethanol and 20 ml of distilled water in the same manner; the supernatant solutions being combined. The remaining insoluble material was washed into a weighed aluminum dish and dried in an oven at 100°.

Lipid Fraction:

The combined supernatant solutions from the three extractions were evaporated to dryness under reduced pressure at 40° in a Buchler flash-evaporator. In order to remove lipid materials, 10 ml of diethyl ether were added to the flask and the resulting ether solution was washed with three 8 to 10 ml portions of distilled water. The substances remaining in ether solution constitute the lipid fraction; the aqueous solution was further resolved by ion exchange chromatography (Canvin and Beevers 1961, Hirs *et al* 1954).

Preparation of Ion Exchange Resins:

The constricted end of a glass column (1.5 × 13 cm) was plugged with glass wool and a slurry of Bio.Rad ion exchange resin, supplied by California Corporation for Biochemical Research, was added to a final depth of 4 cm.

The cation exchange resin, Dowex AG 50W-X8 (200 to 400

mesh) in the hydrogen form, was washed with distilled water until the effluent was neutral.

The Dowex AG I-X10 anion exchange resin (200 to 400 mesh) was supplied in the chloride form. To convert it to formate (and acetate) forms, a IM solution of sodium formate (or sodium acetate) was passed through the resin until the effluent showed a negative chloride test (using $Ag^+$). The column was then washed with 50 ml of a 0.IN solution of HCOOH (or $CH_3COOH$) and finally with distilled water until neutral.

Ethanol/Water Soluble Fractions:

The aqueous solution was reduced to a volume of 5 ml and run onto a neutral column of Dowex AG 50W-X8 cation exchange resin in the hydrogen form. Uncharged compounds and negatively charged (acidic) compounds, principally organic acids with phosphate esters and nucleotides, were washed through with 50 ml of distilled water. Positively charged (basic) compounds, chiefly amino acids, which were adsorbed on the resin were eluted with 50 ml of 2N $NH_4OH$ solution.

Further separations were made using the Dowex AG I-X10 anion exchange resin converted from the chloride form to formate and acetate forms. The effluent from the hydrogen column (aqueous solution of neutral and acidic compounds) was reduced in volume to 5 ml and poured onto a neutral anion column in the formate form. After washing with 50 ml of distilled water, the effluent contained neutral (uncharged)

compounds, and the negatively charged compounds adsorbed on the resin, the organic acid fraction, were displaced with 50 ml of 8N HCOOH solution.

The eluate from the hydrogen column (in 2N $NH_4OH$ solution) was evaporated to dryness, redissolved in distilled water and passed onto a neutral anion column in the acetate form. Neutral and basic amino acids and amides passing through the column in the effluent were completely removed with 50 ml of distilled water, and the acidic amino acids adsorbed on the resin were eluted with 50 ml of 8N $CH_3COOH$ solution.

Amides were removed from the neutral and basic amino acids by hydrolysis to their corresponding acidic amino acids in acid solution (final concentration 2N HCl) placed in a boiling water bath for 4 hours. These acidic amino acids were separated on a neutral Dowex anion exchange column (acetate) as described above. Neutral and basic amino acids were collected in the aqueous effluent. The eluate contained glutamate and aspartate derived from glutamine and asparagine in 8N $CH_3COOH$ solution.

Samples in acid solutions were evaporated to dryness, blown with an air jet to remove all traces of the eluting acid and redissolved in distilled water.

Insoluble Residue and $CO_2$:

A small aliquot (approximately 1 mg) of the insoluble residue, thoroughly dried at 100°, was completely oxidized to $CO_2$ using a mixture of 2 to 3 mg of a finely ground

powder of $KIO_3$ and $K_2Cr_2O_7$ (2:1 w/w) with 7 ml of a 0.07M solution of $KIO_3$ in a solvent containing equal volumes of concentrated $H_2SO_4$ and $H_3PO_4$, using a modification of the method of Van Slyke and Folch (1940) in the apparatus of Stutz and Burris (1951). The $CO_2$ was collected in a 20% (w/v) KOH solution and precipitated as $BaCO_3$ by addition of excess 10% (w/v) aqueous $BaCl_2$.

The $BaCO_3$, both from oxidation of insoluble residue and from $CO_2$ released during the course of the experiment, was filtered onto weighed glass fibre filter paper discs, which were mounted on metal planchets and dried thoroughly at 100°.

### *Analytical Methods*

In order to separate components of the major fractions, the techniques of descending paper chromatography, radioautography, and gradient elution were used. Radioactive compounds and authentic compounds as markers were applied to Whatman No. 1 chromatography paper. After development in the appropriate solvents, markers were detected by characteristic sprays (Smith 1960) and radioactive areas located on one-dimensional chromatograms by a 4 Pi Actigraph II chromatogram scanner (Nuclear-Chicago Corporation, Model 1032) using 3.8 cm strips, and on two-dimensional chromatograms by radioautography using Kodak No Screen medical X-ray film. These areas were eluted by the method of Stiller (1959) and assayed for radioactivity as described above. The procedure for gradient elution is

described with the resolution of the organic acid fraction.

Neutral Fraction:

Paper chromatograms were developed in 1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) for 18 hours at 25°. Markers were located using a spray of 0.5% (w/v) benzidine in ethanol:glacial $CH_3COOH$:40% (w/v) aqueous $CCl_3COOH$ (8:1:1 v/v/v). Radioactive compounds not corresponding to sugars were tested for the presence of acetoin using the technique of Westerfield (1945).

Acidic Amino Acid Fraction:

Phenol:water (8:3 v/v) was found to give the best separation of glutamate and aspartate. Chromatograms were developed for 24 hours at 25°. The markers were sprayed with a solution of 0.1% (w/v) ninhydrin in 1-butanol.

Amide Fraction:

The hydrolyzed amides were chromatographed using the techniques described for acidic amino acids.

Neutral and Basic Amino Acid Fraction:

Neutral and basic amino acids were separated by developing chromatograms in 1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v). Marker amino acids were detected by spraying with 0.1% (w/v) ninhydrin in 1-butanol.

Organic Acid Fraction:

The organic acids were partially resolved by gradient elution based on the method of Palmer (1955). The acids were adsorbed on a 1 × 11 cm column of neutral Dowex anion resin in the formate form. The lower reservoir (the eluting

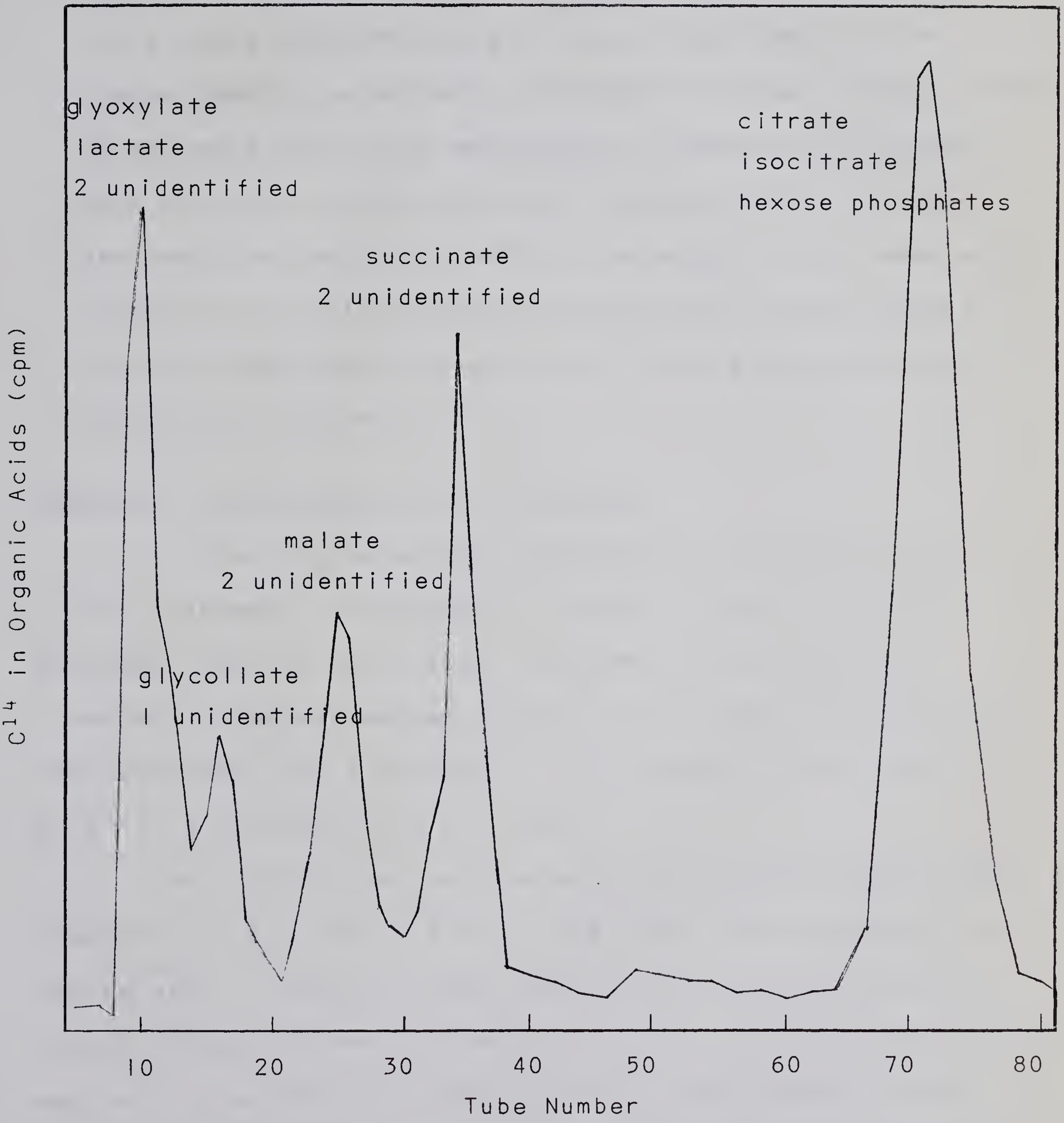
solution), in which homogeneity was maintained by a magnetic stirrer, contained initially 470 ml of distilled water; the upper reservoir contained 8N HCOOH solution. As elution proceeded at a rate of 1 ml/min, the outflow of the eluting solution was balanced by the inflow of HCOOH. Thus the volume remained constant and the acidity constantly increased; the organic acids were serially displaced from the column. The eluate was collected in 2.5 ml fractions using a Buchler fraction collector with a photoelectric volumetric dispensing head and aliquots were removed for detection of radioactivity. After 100 tubes had been collected the resin was washed with 8N HCOOH solution to displace any acids remaining on the column.

Graphs of tube number *vs.* radioactivity showed several consistent peaks (Figure 4). The contents of the tubes of each peak were combined, evaporated to dryness, blown with an air jet to remove all HCOOH and redissolved in water. Chromatograms of the peaks were run in one dimension with 1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) and ethanol: concentrated $NH_3$:water (8:1:1 v/v/v) and in two dimensions using 1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) with either ethanol:concentrated $NH_3$:water (8:1:1 v/v/v) or phenol:water (8:3 v/v). Individual acids were identified by co-chromatography with authentic organic acids. Markers were located using 0.04% (w/v) chlorophenol red in ethanol adjusted to pH 8 (Jones *et al* 1953).

FIGURE 4

CHARACTERISTIC GRADIENT ELUTION

OF THE LABELLED ORGANIC ACID FRACTION



*This graph does not represent any particular experiment, but rather the overall pattern of all the experiments, showing the individual organic acids which accumulated significant amounts of* $C^{14}$. *It does not in any way represent quantitative results.*

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

The individual pool sizes of the organic acids present in the cotyledons were determined after the 18 hour soaking period and at 24 hour intervals thereafter for 3 days. Using a 20 g tissue sample, the cotyledons were sliced, killed, and extracted as previously described for the tissue feeding experiments and proportionately larger amounts of solvents and resins were used to separate the organic acid fraction. After gradient elution of this fraction and complete removal of HCOOH, the acids in the tubes were dissolved in 1 ml of $CO_2$-free water and titrated against standard NaOH solution using 0.1% (w/v) phenolphthalein in ethanol as indicator.

### *Enzymatic Decarboxylation of Glutamate*

In order to determine the amounts of radioactivity in C-1 of glutamate, L-glutamate 1-carboxy-lyase 4.1.1.15 was obtained from the Nutritional Biochemicals Corporation, Cleveland. The enzyme was stored at -10° and just prior to the experiment was dissolved in 0.1N acetate buffer (pH 5.0) at 2° to a concentration of 2 mg/ml.

The reaction was carried out in a sealed Warburg flask (capacity 17 ml) with 0.3 ml of 20% (w/v) KOH solution in the centre well. The main compartment of the flask contained a 0.5 ml aliquot of the glutamate-$C^{14}$ and 0.2 ml (10 µmoles) of carrier L-glutamic acid with 0.5 ml of the buffered enzyme solution in the side arm. The reaction mixture was equilibrated for 5 minutes in a water bath at 37° before the contents of the

side arm were tipped into the flask. After 1 hour the reaction was stopped by addition of absolute ethanol and the absorbed $CO_2$ was converted to $BaCO_3$ and assayed for radioactivity as described previously.

## Enzyme Studies

For the assay of each enzyme 10 g of cotyledons from which testas and embryos had been removed were ground in 10 to 20 ml of buffer solution at 0° in a chilled mortar. The homogenate was passed through 4 layers of cheesecloth and centrifuged at 10000 × *g* for 20 minutes at 2° to remove starch and cellular debris. The specific amounts of the various substrates and cofactors are given in the Results section. Protein concentration was determined from absorbance at 260 and 280 mμ by the method of Warburg and Christian as described by Layne (1957).

Spectrophotometric determinations were made on a double beam spectrophotometer (Beckman Instruments Incorporated, Model DB) using rectangular silica cells with a 1 cm light path. The reference cell contained all the reagents of the sample cell with the exception of substrate.

### Alcohol:$NAD^+$ Oxidoreductase 1.1.1.1

The cotyledons were ground in 0.1M phosphate buffer (pH 9.1) and due to the extreme activity of the enzyme the supernatant solution after centrifugation was diluted tenfold with ice-cold buffer. Oxidation of ethanol was measured by

the increase of optical density at 340 mμ with reduction of $NAD^+$ as described by Racker (1950a).

Citrate (Isocitrate) Hydro-Lyase 4.2.1.3

Both the enzyme and substrate were made up in phosphate buffer (pH 7.4), the enzyme in 0.1M $Na_2HPO_4$ and the substrate, 0.03M trisodium citrate, in 0.05M $Na_2HPO_4$. Enzyme activity was measured by the increase in optical density at 240 mμ due to formation of cis-aconitate (Racker 1950b).

$D_S$-Isocitrate:$NAD^+$ Oxidoreductase (Decarboxylating) 1.1.1.41

The enzyme activity was measured by reduction of $NAD^+$ as in alcohol:$NAD^+$ oxidoreductase. The enzyme was suspended in 0.1M tris buffer (pH 7.4) with 0.1M trisodium isocitrate as substrate, using a slight modification of the technique of Davies (1955).

L-Glutamate:$NAD^+$ Oxidoreductase (Deaminating) 1.4.1.2

The cotyledons were ground in 0.1M phosphate buffer (pH 7.6) for determination of the enzyme by conversion of α-ketoglutarate-5-$C^{14}$ to radioactive glutamate, and by the oxidation of NADH at 340 mμ (based on the method of Olson and Anfinsen 1952), both in the presence of $NH_4^+$. Enzyme suspensions were dialyzed at 0° for 36 hours against distilled water.

L-Amino Acid:2-Oxoglutarate Aminotransferase 2.6.1.-

The enzyme was taken up in 0.1M phosphate buffer (pH 7.1) and dialyzed against distilled water. Activity was measured by the conversion of α-ketoglutarate-5-$C^{14}$ to glutamate-

5-$C^{14}$ in the presence of various amino acid donors and pyridoxal-5-phosphate (Metzler *et al* 1954).

# RESULTS

Gradient elution of 20 g samples of cotyledons showed that the total organic acid content did not appreciably change during the first 4 days of germination, in marked contrast to that of amino acids. The citrate pool was by far the largest throughout this period; both succinate and malate were present in small amounts; α-ketoglutarate, fumarate, and oxaloacetate were not detected.

## Incorporation Sequences

Any significant dilution effect of a large citrate pool would initially influence the metabolism of glycolytic products (*i.e.* ethanol and lactate) labelled with $C^{14}$ more than would the glutamate pool. The ultimate effect would, however, depend upon the relative sizes of the pools and the extent to which they were in equilibrium with the TCA cycle. Ethanol-$C^{14}$ and lactate-$C^{14}$ were presumably converted to acetyl CoA which was principally incorporated into the acids of the TCA cycle. The extent to which lactate participates in the cycle can only be inferred from these experiments since lactate-1-$C^{14}$ gives rise to unlabelled acetyl CoA. Nevertheless, a significantly smaller proportion of the supplied lactate was converted to acetyl CoA due to formation of alanine-$C^{14}$.

Ethanol-$C^{14}$ did not produce appreciable amounts of glutamate-$C^{14}$ until citrate was already heavily labelled. The accumulation of $C^{14}$ in glutamate and its derivatives

(glutamine and γ-aminobutyrate) suggests the ultimate importance of the glutamate pool. Little $C^{14}O_2$ was evolved. These and the subsequent results are considered in detail in the following sections.

Additional support was lent to the operation of this sequence by supplying acetaldehyde, a proposed intermediate of ethanol metabolism, and acetate, a compound closely related to ethanol, both labelled with $C^{14}$, to cotyledon slices. The same trends of incorporation were observed to occur.

In order to investigate further the importance of the glutamate pool on incorporation of radioactive metabolites, labelled isocitrate, α-ketoglutarate, and glutamate itself were supplied to cotyledon slices for 6 hours. These compounds were increasingly incorporated into glutamine-$C^{14}$ and γ-aminobutyrate-$C^{14}$ while still labelling the organic acids to a significant extent.

### *Metabolism of Ethanol, Acetaldehyde, and Acetate*

The patterns of incorporation of the two-carbon compounds were similar. After 5 minutes the largest amounts of $C^{14}$ were in citrate-isocitrate, with slightly less in glutamate. Their positions were reversed after 15 minutes, however, and glutamate persisted as the most heavily labelled single compound during the remainder of the 60-minute experiments. The progressive accumulation of $C^{14}$ in the organic acids of the TCA cycle corresponded to a relative decrease in comparison with the total incorporation into all fractions.

This decline was most apparent with citrate-isocitrate and much less so with succinate and malate. None of the other cycle acids accumulated detectable $C^{14}$ although a large number of acidic compounds not directly participating in the cycle became increasingly labelled, notably hexose phosphates (Figure 12).

Fewer nitrogenous compounds accumulated $C^{14}$. Glutamate was by far the most important of these but labelling was also detected in aspartate; in glutamine of the amide fraction; and in homoserine, with traces of γ-aminobutyrate appearing in 60 minutes, in the neutral and basic amino acid fraction. Although the arginine pool is large, no radioactive arginine was found. The rate of $C^{14}$ accumulation in the neutral and basic amino acids and aspartate paralleled more or less that of total incorporation while that into amides increased strikingly.

There was a general trend of increased incorporation of $C^{14}$ into the lipid fraction and the insoluble fraction (polypeptides, protein, and probably some polysaccharides). C-1 was a significantly better precursor of $CO_2$ than C-2.

In spite of the general similarity of these trends there were characteristic features of incorporation of $C^{14}$, the most notable of which were the products and extent of accumulation in the neutral fraction.

Ethanol:

The neutral fraction in the ethanol experiments con-

sisted of a single unidentified compound which is not a sugar. It showed a decreasing rate of incorporation similar to that of the organic acid fraction. In addition, aspartate was more heavily labelled than succinate but less than malate in 5 minutes, suggesting at least one full turn of the TCA cycle. The results of the ethanol-1-$C^{14}$ and ethanol-2-$C^{14}$ incorporation, shown in Tables 1 and 2, represent an average of 2 independent series of experiments; the salient features are summarized in Figures 5 and 6.

Acetaldehyde:

The same neutral compound produced by ethanol accumulated $C^{14}$ in much greater amounts from acetaldehyde-$C^{14}$ (Table 3). Such a significant proportion of the acetaldehyde was diverted to this substance that the incorporation pattern into the other fractions appeared altered (Figure 7) although the distribution closely resembled that of ethanol. A singular difference was the very low rate of $C^{14}$ incorporation into lipids.

Acetate:

The neutral fraction extracted after supplying acetate-$C^{14}$ contained labelled sugars (principally glucose) and 3 unidentified non-sugar compounds. One of these compounds contained more $C^{14}$ initially but after 60 minutes barely detectable amounts of radioactivity remained (Tables 4 and 5). Only acetate-1-$C^{14}$ labelled more than trace amounts of the other two, one of which was the same compound produced by

# TABLE I

## INCORPORATION OF ETHANOL-1-$C^{14}$

| Fraction | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 2900 | *3.2* | 9700 | *3.3* | 25100 | *3.7* | 43300 | *4.4* |
| Neutral | | | | | | | | |
| sugars | not detected | | not detected | | not detected | | trace | *0.0* |
| others | 5800 | *6.4* | 17000 | *5.8* | 24900 | *3.6* | 35300 | *3.6* |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 20600 | *22.8* | 107900 | *36.6* | 273400 | *39.7* | 329300 | *33.2* |
| aspartate | 6700 | *7.4* | 24600 | *8.3* | 63700 | *9.3* | 83900 | *8.5* |
| Amide | 1300 | *1.5* | 6500 | *2.2* | 30100 | *4.4* | 56000 | *5.6* |
| Neutral and Basic Amino Acid | 3000 | *3.3* | 9300 | *3.2* | 20100 | *2.9* | 34100 | *3.4* |
| Organic Acid | 47700 | *52.8* | 112600 | *38.2* | 221300 | *32.2* | 318600 | *32.1* |
| Carbon Dioxide | 400 | *0.5* | 2700 | *0.9* | 12600 | *1.8* | 52000 | *5.2* |
| Insoluble | 1900 | *2.1* | 4400 | *1.5* | 16800 | *2.4* | 40000 | *4.0* |
| Total $C^{14}$ Incorporated | 90300 | | 294700 | | 688000 | | 992500 | |

*0.5 g of slices incubated with 5.0 μc/1.0 μmole of Ethanol-1-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

## TABLE 2

## INCORPORATION OF ETHANOL-2-$C^{14}$

| Fraction | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 2900 | *4.5* | 8500 | *4.4* | 23000 | *5.1* | 52900 | *6.4* |
| Neutral | | | | | | | | |
| sugars | not detected | | not detected | | not detected | | trace | *0.0* |
| others | 3800 | *5.9* | 10500 | *5.5* | 18000 | *3.9* | 32400 | *3.9* |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 11300 | *17.5* | 58500 | *30.4* | 157300 | *34.8* | 232700 | *28.0* |
| aspartate | 3700 | *5.7* | 7200 | *3.7* | 20800 | *4.6* | 49000 | *5.9* |
| Amide | 1000 | *1.6* | 6900 | *3.6* | 34300 | *7.6* | 72000 | *8.7* |
| Neutral and Basic Amino Acid | 2500 | *3.9* | 7800 | *4.0* | 9900 | *2.2* | 24600 | *3.0* |
| Organic Acid | 35000 | *54.3* | 86800 | *45.1* | 171400 | *37.9* | 304100 | *36.7* |
| Carbon Dioxide | 100 | *0.2* | 200 | *0.1* | 300 | *0.1* | 2600 | *0.3* |
| Insoluble | 4100 | *6.4* | 6200 | *3.2* | 17300 | *3.8* | 58900 | *7.1* |
| Total $C^{14}$ Incorporated | 64400 | | 192600 | | 452300 | | 829200 | |

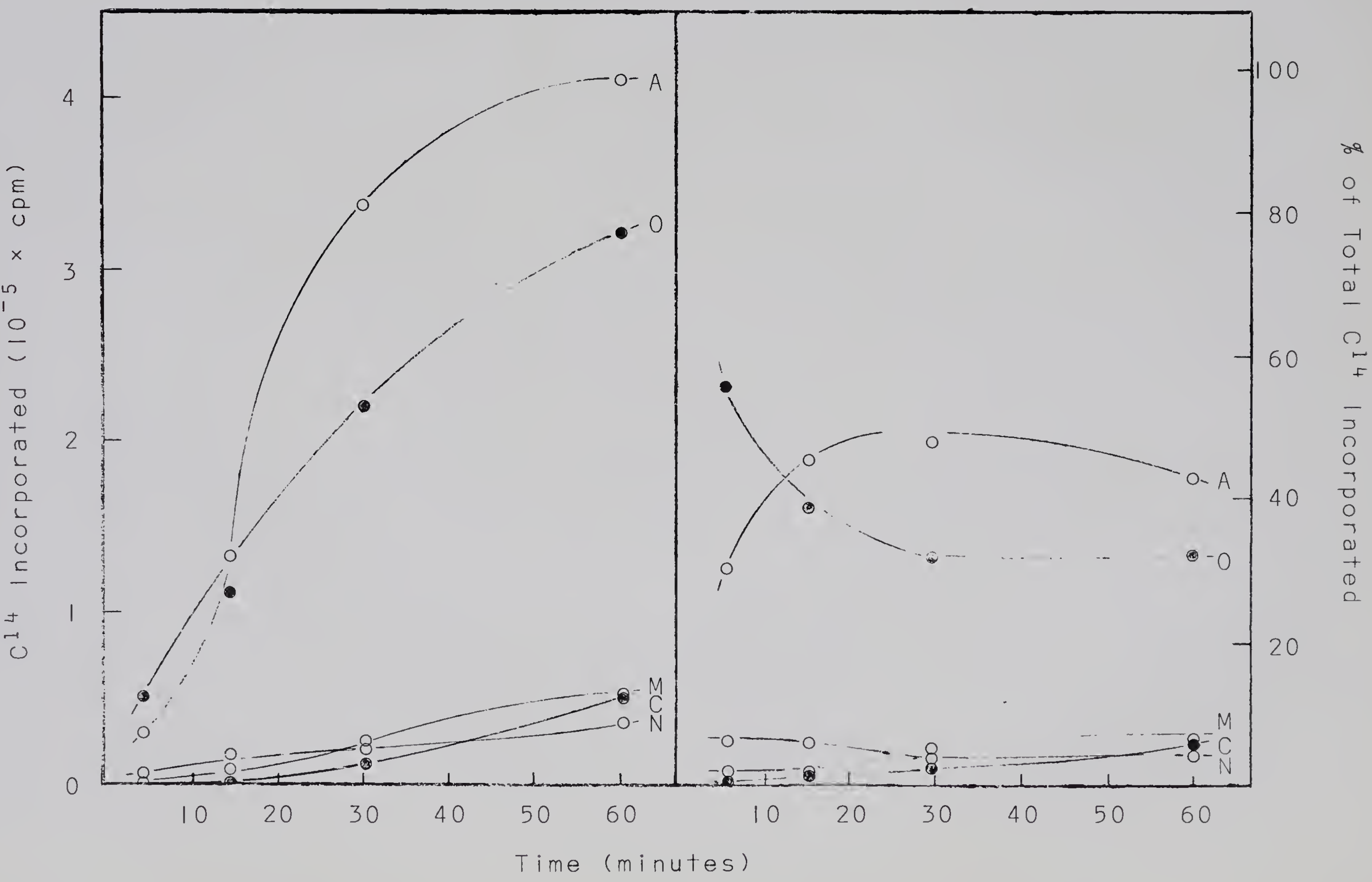
*0.5 g of slices incubated with 5.0 μc/1.0 μmole of Ethanol-2-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

Data from Table 1

| | |
|---|---|
| A | acidic amino acid fraction |
| C | carbon dioxide fraction |
| M | amide fraction |
| N | neutral fraction |
| O | organic acid fraction |

FIGURE 5

INCORPORATION OF C$^{14}$ FROM ETHANOL-1-C$^{14}$



Data from Table 2

A acidic amino acid fraction

C carbon dioxide fraction

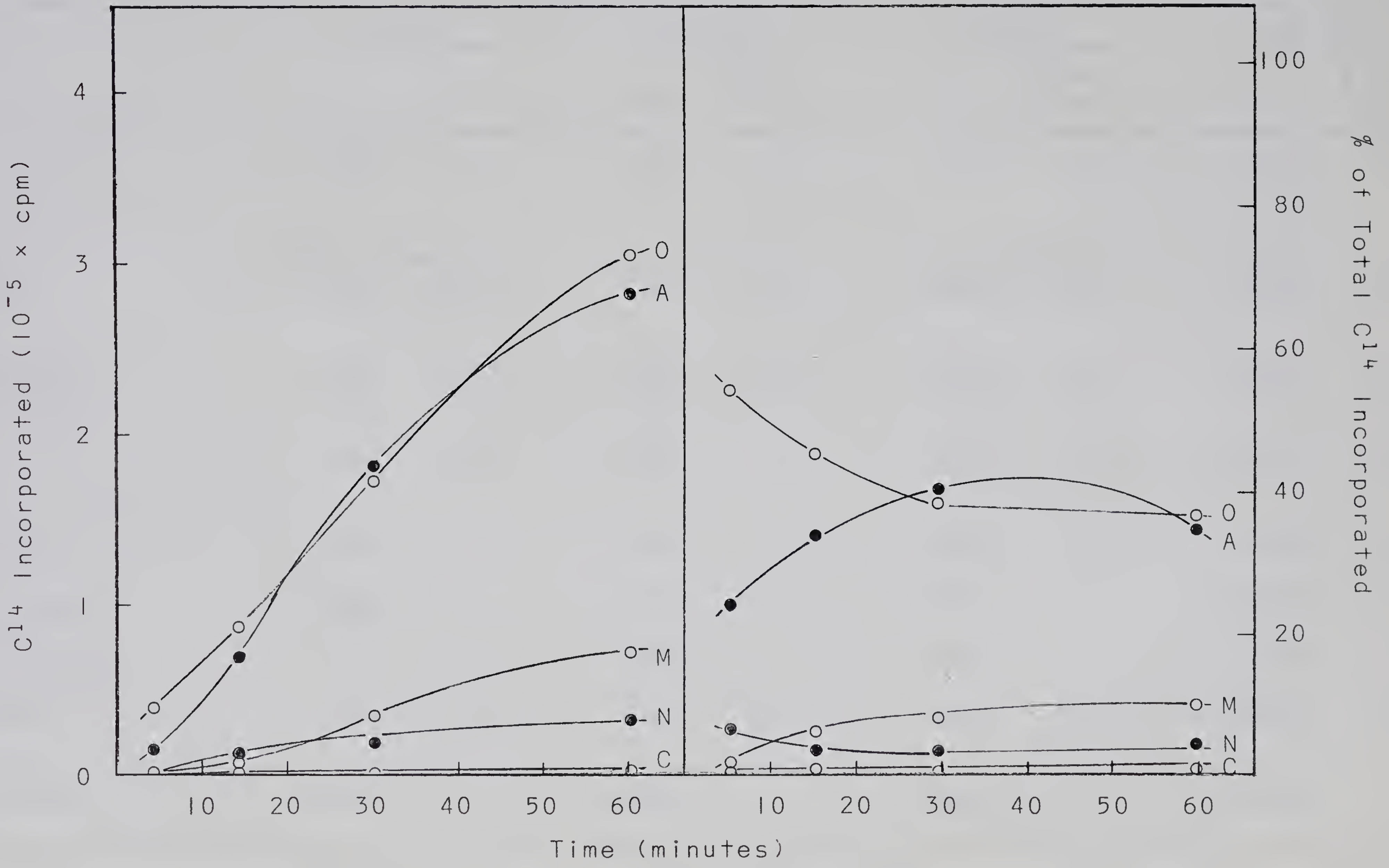
M amide fraction

N neutral fraction

O organic acid fraction

FIGURE 6

INCORPORATION OF $C^{14}$ FROM ETHANOL-2-$C^{14}$



# TABLE 3

## INCORPORATION OF ACETALDEHYDE-1,2-$C^{14}$

| Fraction | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 900 | *3.7* | 1300 | *2.7* | 2300 | *2.0* | 3400 | *1.6* |
| Neutral | | | | | | | | |
| sugars | not detected | | not detected | | not detected | | trace | *0.0* |
| others | 5900 | *24.4* | 8100 | *16.7* | 13900 | *12.1* | 19500 | *9.4* |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 3700 | *15.3* | 9500 | *19.6* | 37100 | *32.4* | 49500 | *23.9* |
| aspartate | 600 | *2.5* | 1200 | *2.5* | 5100 | *4.5* | 11900 | *5.8* |
| Amide | 300 | *1.2* | 2300 | *4.7* | 7200 | *6.3* | 18400 | *8.9* |
| Neutral and Basic Amino Acid | 1700 | *7.0* | 3400 | *7.0* | 3900 | *3.4* | 9000 | *4.4* |
| Organic Acid | 9600 | *39.7* | 19000 | *39.3* | 36500 | *31.8* | 72900 | *35.2* |
| Carbon Dioxide | 200 | *0.8* | 900 | *1.9* | 2800 | *2.4* | 7500 | *3.6* |
| Insoluble | 1300 | *5.4* | 2700 | *5.6* | 5800 | *5.1* | 14900 | *7.2* |
| Total $C^{14}$ Incorporated | 24200 | | 48400 | | 114600 | | 207000 | |

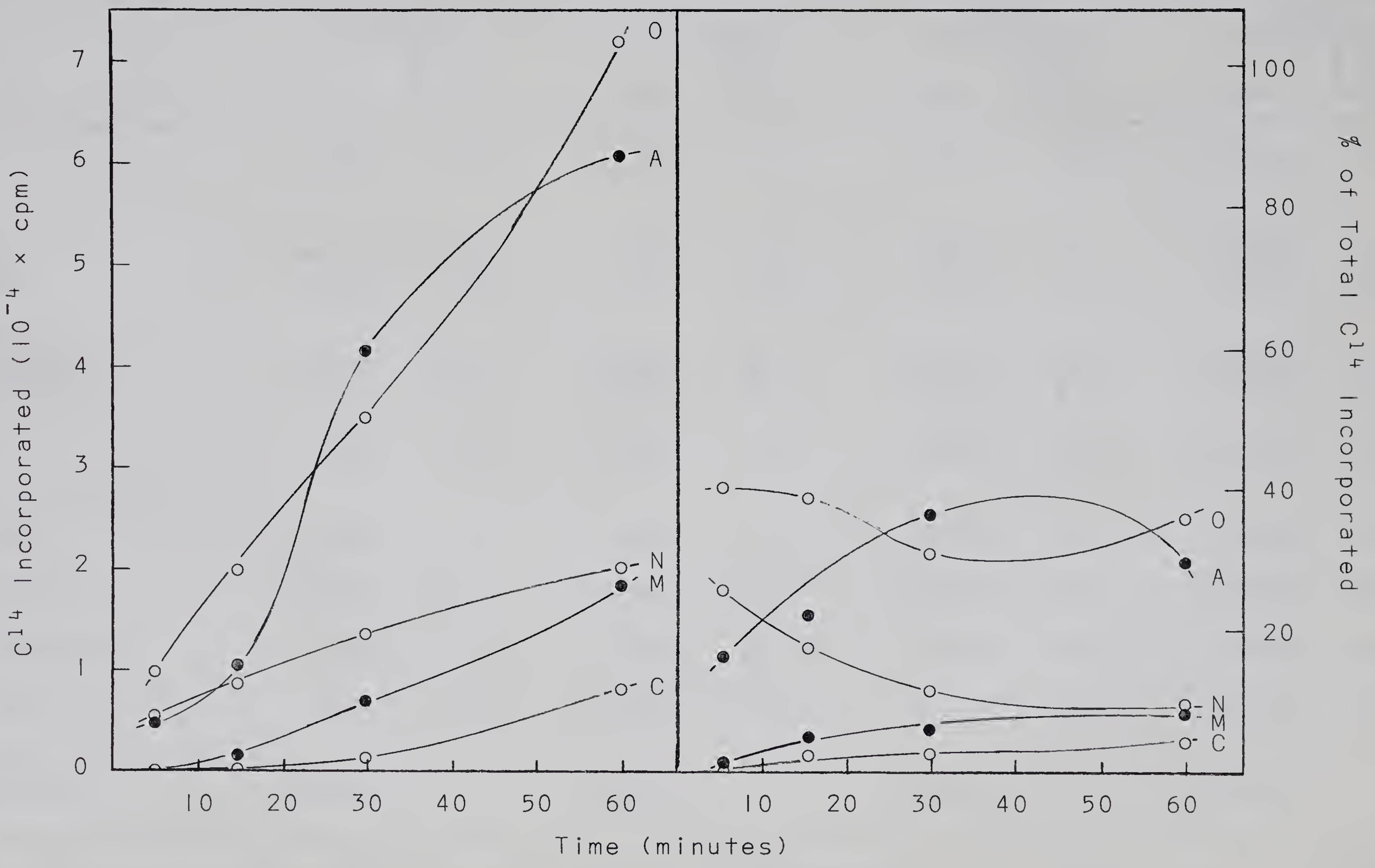
*0.5 g of slices incubated with 2.5 μc/2.5 μmoles of Acetaldehyde-1,2-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

Data from Table 3

| | |
|---|---|
| A | acidic amino acid fraction |
| C | carbon dioxide fraction |
| M | amide fraction |
| N | neutral fraction |
| O | organic acid fraction |

FIGURE 7

INCORPORATION OF $C^{14}$ FROM ACETALDEHYDE-1,2-$C^{14}$



## TABLE 4

## INCORPORATION OF ACETATE-I-$C^{14}$

| Fraction | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 5700 | 3.0 | 20500 | 3.9 | 47000 | 4.9 | 68100 | 5.1 |
| Neutral | | | | | | | | |
| sugars | not detected | | 4700 | 0.9 | 9800 | 1.1 | 20300 | 1.5 |
| others | 6300 | 3.4 | 4900 | 0.9 | 7000 | 0.7 | 3000 | 0.3 |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 46000 | 24.7 | 172800 | 32.6 | 309600 | 32.5 | 386300 | 29.0 |
| aspartate | 11100 | 6.0 | 45600 | 8.6 | 86300 | 9.1 | 85900 | 6.5 |
| Amide | 2600 | 1.4 | 19500 | 3.7 | 38800 | 4.1 | 125100 | 9.4 |
| Neutral and Basic Amino Acid | 2200 | 1.2 | 5700 | 1.1 | 10100 | 1.1 | 23500 | 1.8 |
| Organic Acid | 105100 | 56.4 | 223000 | 42.0 | 356700 | 37.4 | 452000 | 34.0 |
| Carbon Dioxide | 2400 | 1.3 | 17500 | 3.3 | 40200 | 4.2 | 85100 | 6.3 |
| Insoluble | 4800 | 2.6 | 16100 | 3.0 | 47100 | 4.9 | 81100 | 6.1 |
| Total $C^{14}$ Incorporated | 186200 | | 530300 | | 952600 | | 1330400 | |

*0.5 g of slices incubated with 5.0 μc/1.0 μmole of Na Acetate-1-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

## TABLE 5

## INCORPORATION OF ACETATE-2-$C^{14}$

| Fraction | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 5000 | *3.2* | 17200 | *4.0* | 33900 | *3.5* | 41200 | *3.1* |
| Neutral | | | | | | | | |
| sugars | 1700 | *1.1* | 3000 | *0.7* | 27800 | *2.9* | 41400 | *3.1* |
| others | 3600 | *2.3* | 2700 | *0.6* | 6000 | *0.6* | trace | *0.0* |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 25500 | *16.1* | 129900 | *30.1* | 363200 | *37.9* | 398700 | *30.2* |
| aspartate | 5400 | *3.4* | 20400 | *4.7* | 80800 | *8.5* | 97800 | *7.4* |
| Amide | 1500 | *1.0* | 10800 | *2.5* | 49600 | *5.2* | 147900 | *11.2* |
| Neutral and Basic Amino Acid | 2400 | *1.5* | 9100 | *2.1* | 20300 | *2.1* | 52300 | *4.0* |
| Organic Acid | 109400 | *69.3* | 230700 | *53.4* | 346800 | *36.2* | 489400 | *37.0* |
| Carbon Dioxide | 700 | *0.4* | 2000 | *0.5* | 2400 | *0.3* | 6800 | *0.5* |
| Insoluble | 2700 | *1.7* | 6100 | *1.4* | 26800 | *2.8* | 46900 | *3.5* |
| Total $C^{14}$ Incorporated | 157900 | | 431900 | | 957600 | | 1322400 | |

*0.5 g of slices incubated with 5.0 μc/1.0 μmole of Na Acetate-2-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

ethanol and acetaldehyde. Acetate also differed from ethanol in that after 5 minutes, succinate was equally or more heavily labelled than malate and significantly more so than aspartate. This would suggest that label was only beginning to accumulate in the malate pool and therefore that acetate was metabolized less quickly than ethanol. The results of Tables 4 and 5 represent an average of 2 experiments; the prominent features are summarized in Figures 8 and 9.

Greater quantities of acetate-$C^{14}$ were utilized than of the other two-carbon compounds but this might be due volatility at 30°: $CH_3COOH$, at pH 5.5 essentially undissociated, bp 118.1°; $CH_3CH_2OH$ bp 78.5°; $CH_3CHO$ bp 21°.

In an experiment designed to investigate the sequence of $C^{14}$ incorporation from acetate-1-$C^{14}$ over periods of less than 5 minutes, substantial evidence for the operation of the TCA cycle was obtained (Table 6). In 1 minute over 50% of the incorporated label was in citrate-isocitrate and succinate contained more than malate. This relationship did not change appreciably during the course of the experiment (Figures 10 and 11). The only notable rate of increase in $C^{14}$ content was in the acidic amino acid fraction, mainly glutamate-$C^{14}$.

### *Metabolism of Lactate*

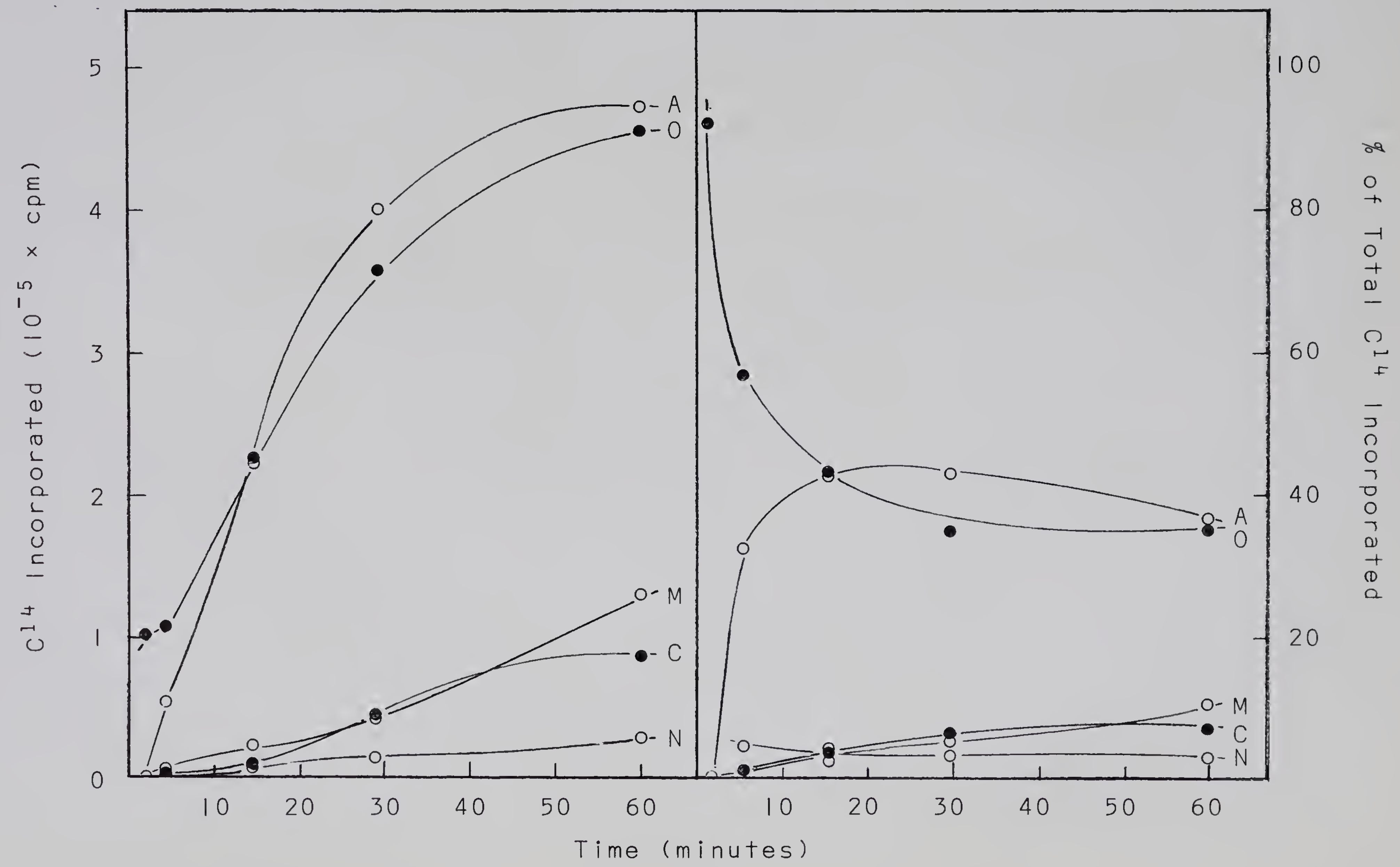
Carboxyl-labelled lactate was used in this experiment and since acetyl CoA arising from it would not be labelled, no $C^{14}$ could be incorporated into the TCA cycle by this means.

Data from Table 4

| | |
|---|---|
| A | acidic amino acid fraction |
| C | carbon dioxide fraction |
| M | amide fraction |
| N | neutral fraction |
| O | organic acid fraction |

FIGURE 8

INCORPORATION OF C$^{14}$ FROM ACETATE-1-C$^{14}$

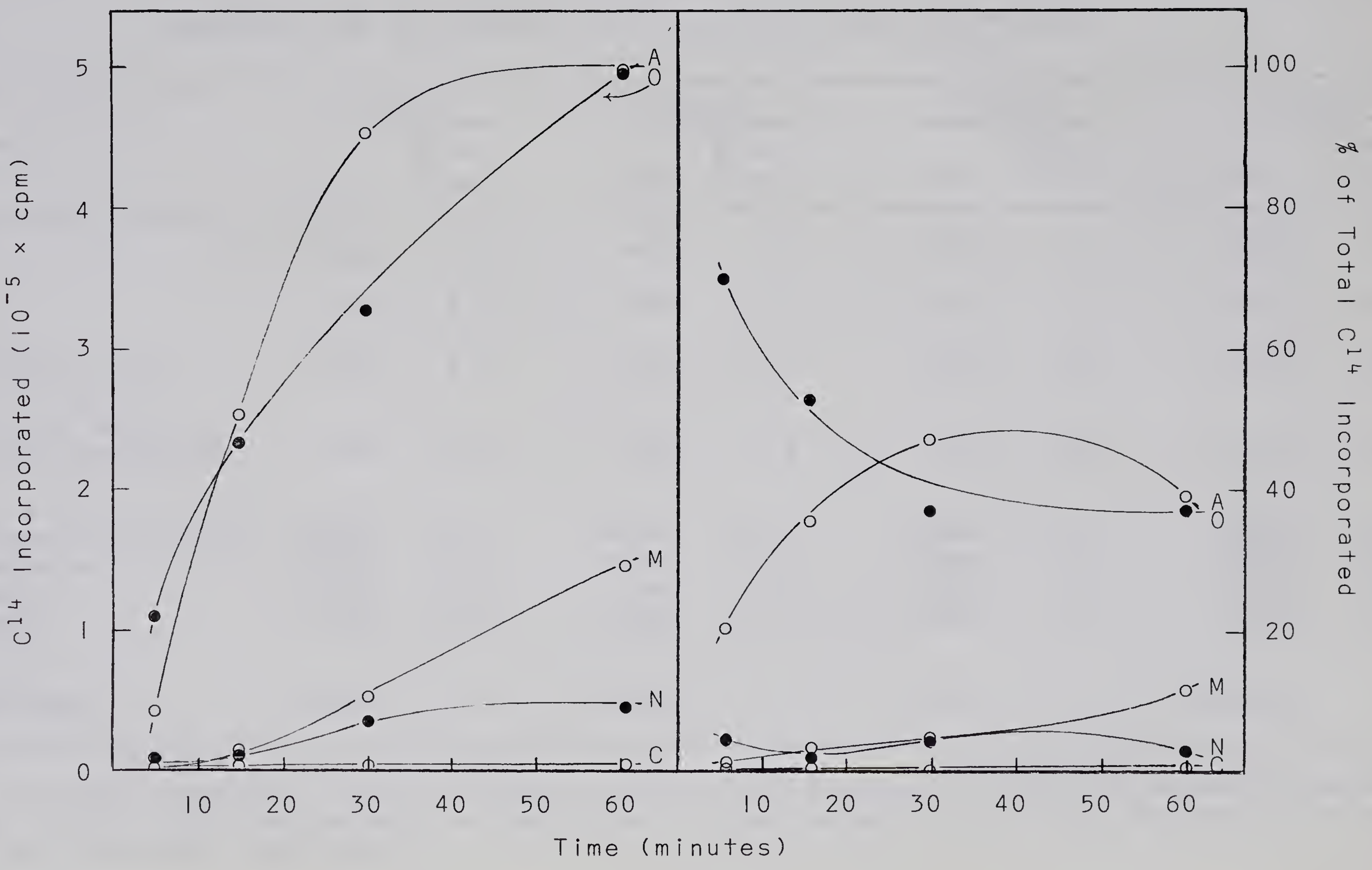


*Data from Table 5*

| | |
|---|---|
| *A* | *acidic amino acid fraction* |
| *C* | *carbon dioxide fraction* |
| *M* | *amide fraction* |
| *N* | *neutral fraction* |
| *O* | *organic acid fraction* |

FIGURE 9

INCORPORATION OF $C^{14}$ FROM ACETATE-2-$C^{14}$



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[illegible]

## TABLE 6

## INCORPORATION OF ACETATE-1-$C^{14}$ IN LESS THAN FIVE MINUTES

| Fraction | 1 min |  | 2 min |  | 3 min |  | 5 min |  |
|---|---|---|---|---|---|---|---|---|
|  | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 1000 | 0.9 | 1600 | 1.3 | 3100 | 2.3 | 3300 | 2.3 |
| Neutral | 1900 | 1.7 | 2000 | 1.7 | 2100 | 1.6 | 3500 | 2.4 |
| Acidic Amino Acid | 1500 | 1.3 | 2100 | 1.7 | 4100 | 3.0 | 10700 | 7.3 |
| Neutral and Basic Amino Acid and Amide | 400 | 0.4 | 600 | 0.5 | 700 | 0.5 | 1100 | 0.7 |
| Organic Acid |  |  |  |  |  |  |  |  |
| citrate/isocitrate | 58300 | 52.7 | 64200 | 52.6 | 72500 | 54.0 | 79200 | 54.2 |
| succinate | 23200 | 21.0 | 26700 | 21.9 | 29800 | 22.2 | 22900 | 15.6 |
| malate | 7200 | 6.5 | 6800 | 5.6 | 5100 | 3.8 | 8300 | 5.8 |
| others | 17100 | 15.5 | 17900 | 14.7 | 16900 | 12.6 | 17100 | 11.7 |
| Total $C^{14}$ Incorporated | 110600 |  | 121900 |  | 134300 |  | 146100 |  |

*0.5 g of slices incubated with 5.0 μc/1.0 μmole of Na Acetate-1-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 25° for time periods as indicated. Total volume 0.6 ml. No determination made of $CO_2$ and Insoluble fractions.*

LABELLED ORGANIC ACIDS IN I MINUTE

*Radioautograph of a two-dimensional paper chromatogram of the organic acid fraction from acetate-1-*$C^{14}$.

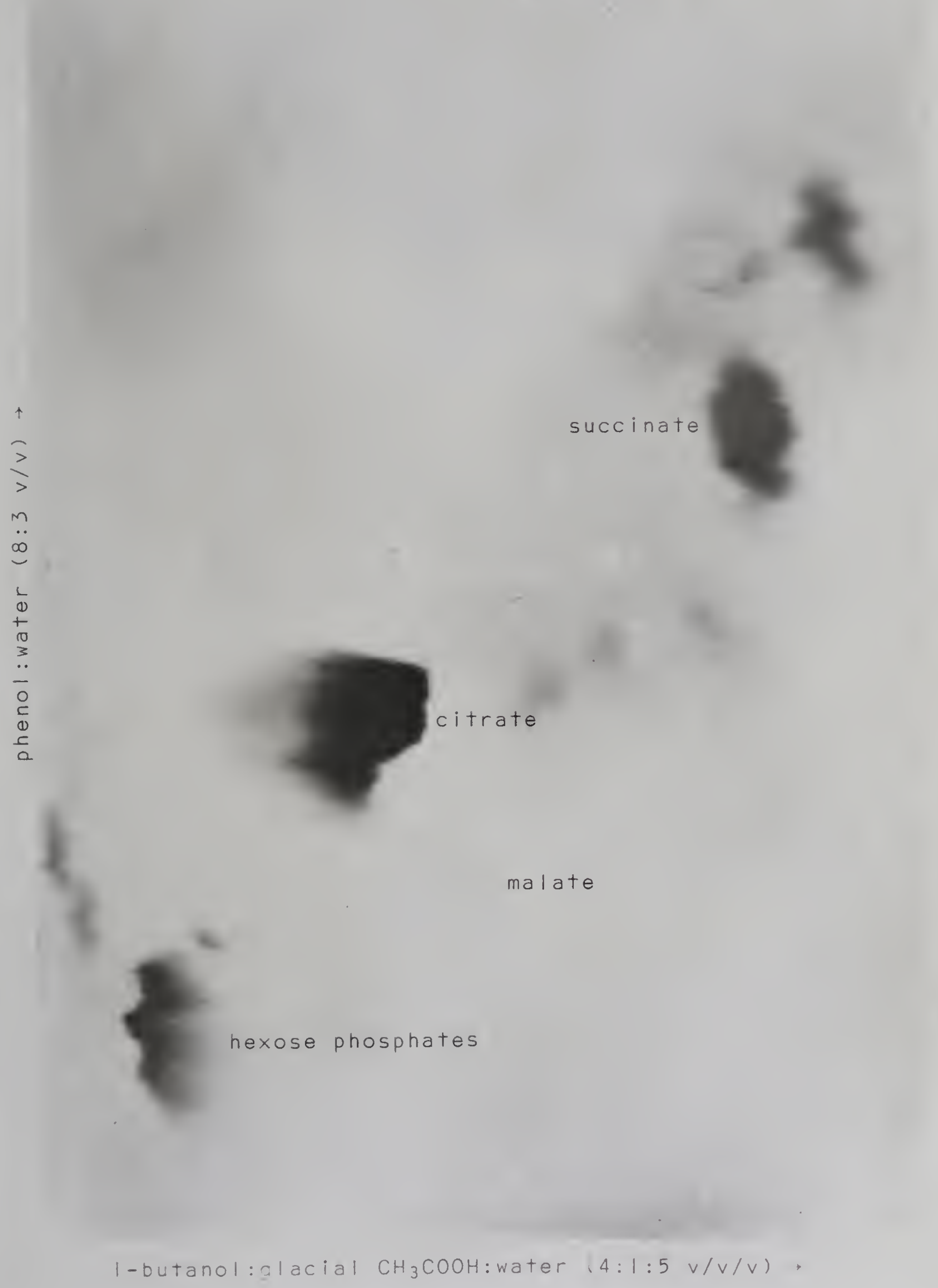
FIGURE 10


phenol:water (8 3 v/v) →
succinate
isocitrate
citrate
n-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) →

LABELLED ORGANIC ACIDS IN 5 MINUTES

*Radioautograph of a two-dimensional paper chromatogram of the organic acid fraction from acetate-1-*$C^{14}$.

FIGURE II


succinate
citrate
malate
hexose phosphates
phenol:water (8:3 v/v) →
1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) →

LABELLED ORGANIC ACIDS IN 60 MINUTES

*Radioautograph of a two-dimensional paper chromatogram of the organic acid fraction from acetate-1-*$C^{14}$.

FIGURE 12


succinate
citrate
malate
hexose phosphates
phenol:water (8:3 v/v) →
1-butanol:glacial $CH_3COOH$:water (4:1:5 v/v/v) →

The fact that the cycle acids and related compounds did become labelled (Table 7) implies $CO_2$ fixation. The characteristic feature of lactate metabolism was the significant incorporation into alanine, which accounted for the total radioactivity in the neutral and basic amino acid fraction except for minute amounts of homoserine-$C^{14}$. Lactate was initially incorporated in large amounts into glucose and sucrose, and glycerol produced from the same sequence could account for the accumulation of $C^{14}$ in the glyceride moiety of lipids since fatty acids would not become labelled.

### *Metabolism of Isocitrate, α-Ketoglutarate, and Glutamate*

The characteristic accumulation of $C^{14}$ in 6-hour experiments was in glutamate and its derivatives and $CO_2$. Sugars were labelled to a much lesser degree than from acetate (Table 8). Label present in the supplied acid at the conclusion of the experiment was not included in the $C^{14}$ content of respective fraction of Table 8.

Isocitrate-5,6-$C^{14}$ was present largely as citrate due to the action of citrate (isocitrate) hydro-lyase, the equilibrium mixture of which is principally citrate (Krebs 1953). Since a good separation of citrate and isocitrate could not be achieved, the radioactivity present in both has been excluded from Table 8. Only 31% of the supplied $C^{14}$ was incorporated into other compounds.

α-Ketoglutarate was readily assimilated, and glutamate, glutamine, and $CO_2$ accounted for nearly 60% of the incorporated

## TABLE 7

## INCORPORATION OF LACTATE-1-$C^{14}$

| Fraction | 30 min | | 120 min | |
|---|---|---|---|---|
| | cpm | % of total | cpm | % of total |
| Lipid | 6900 | *3.6* | 7800 | *2.0* |
| Neutral | | | | |
| sugars | 81600 | *43.0* | 15800 | *4.1* |
| others | not detected | | trace | *0.0* |
| Acidic Amino Acid | | | | |
| glutamate | 3800 | *2.0* | 13800 | *3.6* |
| aspartate | 2600 | *1.4* | 7500 | *1.9* |
| Amide | 1700 | *0.9* | 1600 | *0.4* |
| Neutral and Basic Amino Acid | 43700 | *23.0* | 141900 | *36.5* |
| Organic Acid | 11200 | *5.9* | 16000 | *4.1* |
| Carbon Dioxide | 18700 | *9.9* | 142800 | *36.7* |
| Insoluble | 19500 | *10.3* | 41800 | *10.7* |
| Total $C^{14}$ Incorporated | 189700 | | 389000 | |

*0.5 g of slices incubated with 5.0 μc/1.7 μmoles of Na Lactate-1-$C^{14}$ and 50 μmoles of $NaH_2PO_4$ (pH 5.5) at 30° for time periods as indicated. Total volume 0.6 ml.*

## TABLE 8

## INCORPORATION OF ACIDS ASSOCIATED WITH THE TRICARBOXYLIC ACID CYCLE

| Fraction | Acetate-2-$C^{14}$ | | Isocitrate-5,6-$C^{14}$ | | α-Ketoglutarate-5-$C^{14}$ | | Glutamate-3,4-$C^{14}$ | |
|---|---|---|---|---|---|---|---|---|
| | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 16300 | *5.6* | 800 | *0.2* | 6100 | *0.6* | 3500 | *0.3* |
| Neutral | | | | | | | | |
| sugars | 23600 | *8.2* | 7600 | *2.3* | 32100 | *3.1* | 8600 | *0.7* |
| others | not detected | | not detected | | not detected | | not detected | |
| Acidic Amino Acid | | | | | | | | |
| glutamate | 27700 | *9.6* | 39700 | *11.8* | 115300 | *11.1* | — | |
| aspartate | not detected | | 1600 | *0.5* | 4800 | *0.5* | 22000 | *1.8* |
| others | not detected | | not detected | | 4600 | *0.4* | 48000 | *4.0* |
| Amide | 50100 | *17.3* | 19400 | *5.8* | 146500 | *14.2* | 367900 | *30.7* |
| Neutral and Basic Amino Acid | 14900 | *5.2* | 8500 | *2.5* | 57200 | *5.5* | 232200 | *19.4* |
| Organic Acid | 112000 | *38.7* | 111100 | *33.1* | 308400 | *29.8* | 253400 | *21.1* |
| Carbon Dioxide | 31100 | *10.8* | 106200 | *31.6* | 331200 | *32.0* | 63600 | *5.3* |
| Insoluble | 13400 | *4.6* | 40900 | *12.2* | 28900 | *2.8* | 199900 | *16.7* |
| Total $C^{14}$ Incorporated | 289100 | | 335800 | | 1035100 | | 1199100 | |

*0.5 g of slices incubated with 50 μmoles of* $NaH_2PO_4$ *(pH 5.5) and 2.0 μc/μmole of Na Acetate-2-*$C^{14}$*, 2.0 μc/7.4 μmoles of* $Na_3$*Isocitrate-5,6-*$C^{14}$*, 2.0 μc/μmole of* $Na_2$*α-Ketoglutarate-5-*$C^{14}$*, and 3.0 μc/μmole of Glutamic Acid-3,4-*$C^{14}$ *as indicated for 6 hours. Total volume 0.6 ml.*

$C^{14}$. Glutamate and γ-aminobutyrate accumulated almost one-half of the label from glutamate-3,4-$C^{14}$; the amount of $C^{14}$ traversing the TCA cycle was therefore reduced.

In these 6-hour experiments, acidic amino acids other than aspartate and glutamate became labelled. Although not identified, these acids correspond to the positions on paper chromatograms developed in phenol:water of α-aminoadipate (Hatanaka and Virtanen 1962) and γ-glutamyl alanine (Virtanen and Berg 1954).

## Enzyme Studies

To corroborate the results of the incorporation sequence experiments, the pea cotyledons were examined for the presence of enzymes which were capable of catalyzing the reactions. Four such enzymes in the sequence of incorporation of ethanol into glutamate were found to be present. These enzymes and the reactions they catalyze are:

alcohol:$NAD^+$ oxidoreductase 1.1.1.1

$$\text{ethanol} + NAD^+ \rightleftharpoons \text{acetaldehyde} + NADH + H^+$$

citrate (isocitrate) hydro-lyase 4.2.1.3

$$\text{citrate} \rightleftharpoons \text{carbonium ion complex (Figure 3)} \rightleftharpoons \text{isocitrate}$$

threo-$D_S$-isocitrate:$NAD^+$ oxidoreductase (decarboxylating) 1.1.1.41

$$\text{isocitrate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + CO_2 + NADH + H^+$$

L-glutamate:NAD$^+$ oxidoreductase (deaminating) 1.4.1.2

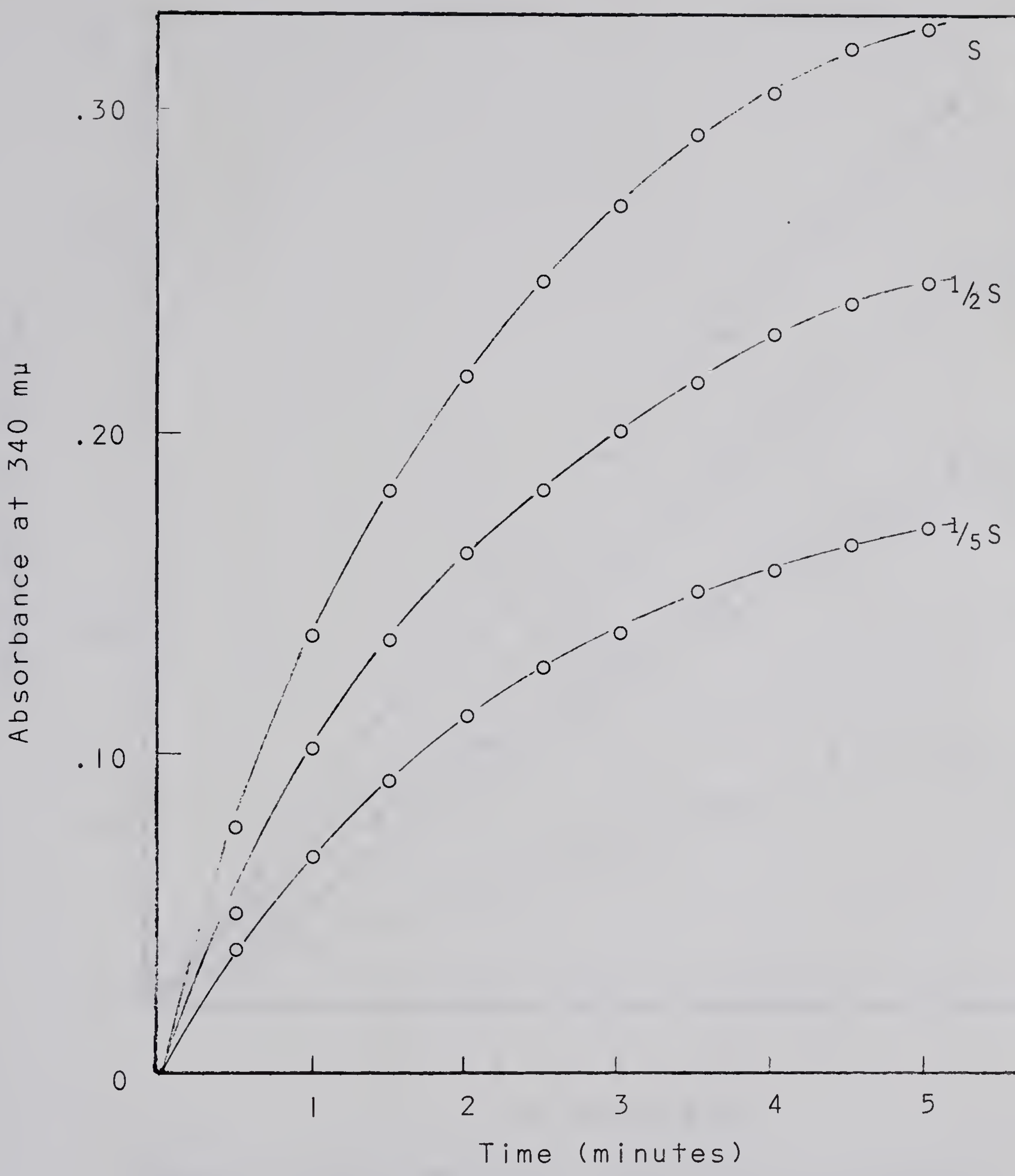
$$\alpha\text{-ketoglutarate} + NH_4 + NADH \rightleftharpoons \text{glutamate} + NAD^+$$

Figures 13 to 16 show the effects of enzyme and substrate concentrations on reaction rates.

In addition transamination reactions between several amino acids and α-ketoglutarate were shown (Table 9). The specific activities of these enzymes are listed in Table 10.

FIGURE 13

EFFECT OF SUBSTRATE CONCENTRATION ON ALCOHOL:NAD$^+$ OXIDOREDUCTASE ACTIVITY



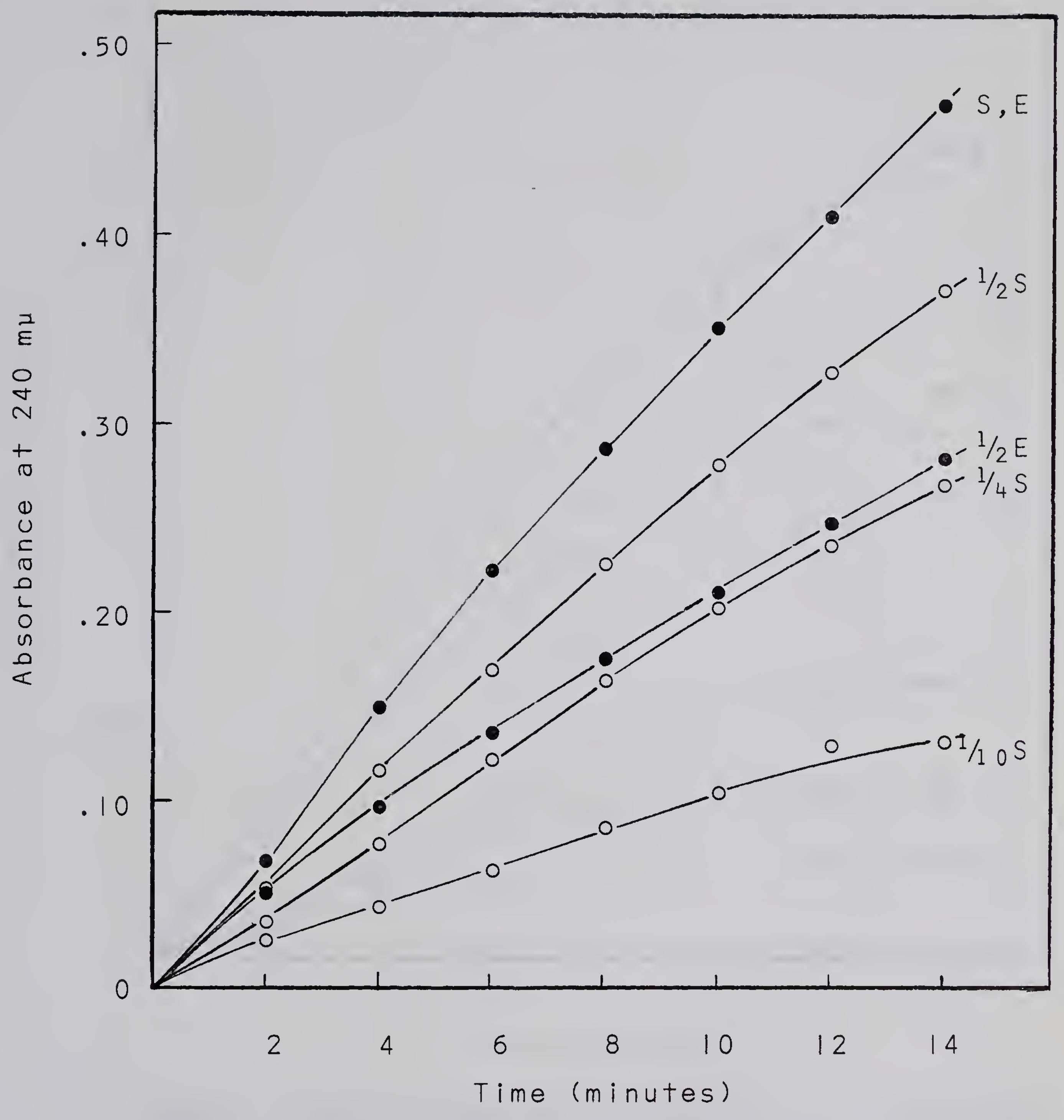
*Sample cells contained 50 μmoles of $Na_2HPO_4$ buffer (pH 7.4) and 0.8 μmoles of NAD+:*
*with enzyme suspension (0.5 mg protein) + Ethanol,*
*S 150 μmoles*
*$^1/_2$S 75 μmoles*
*$^1/_5$S 30 μmoles*
*Total volume 3.0 ml.*

[illegible]

[illegible]

FIGURE 14

EFFECTS OF SUBSTRATE AND ENZYME CONCENTRATIONS ON CITRATE (ISOCITRATE) HYDRO-LYASE ACTIVITY

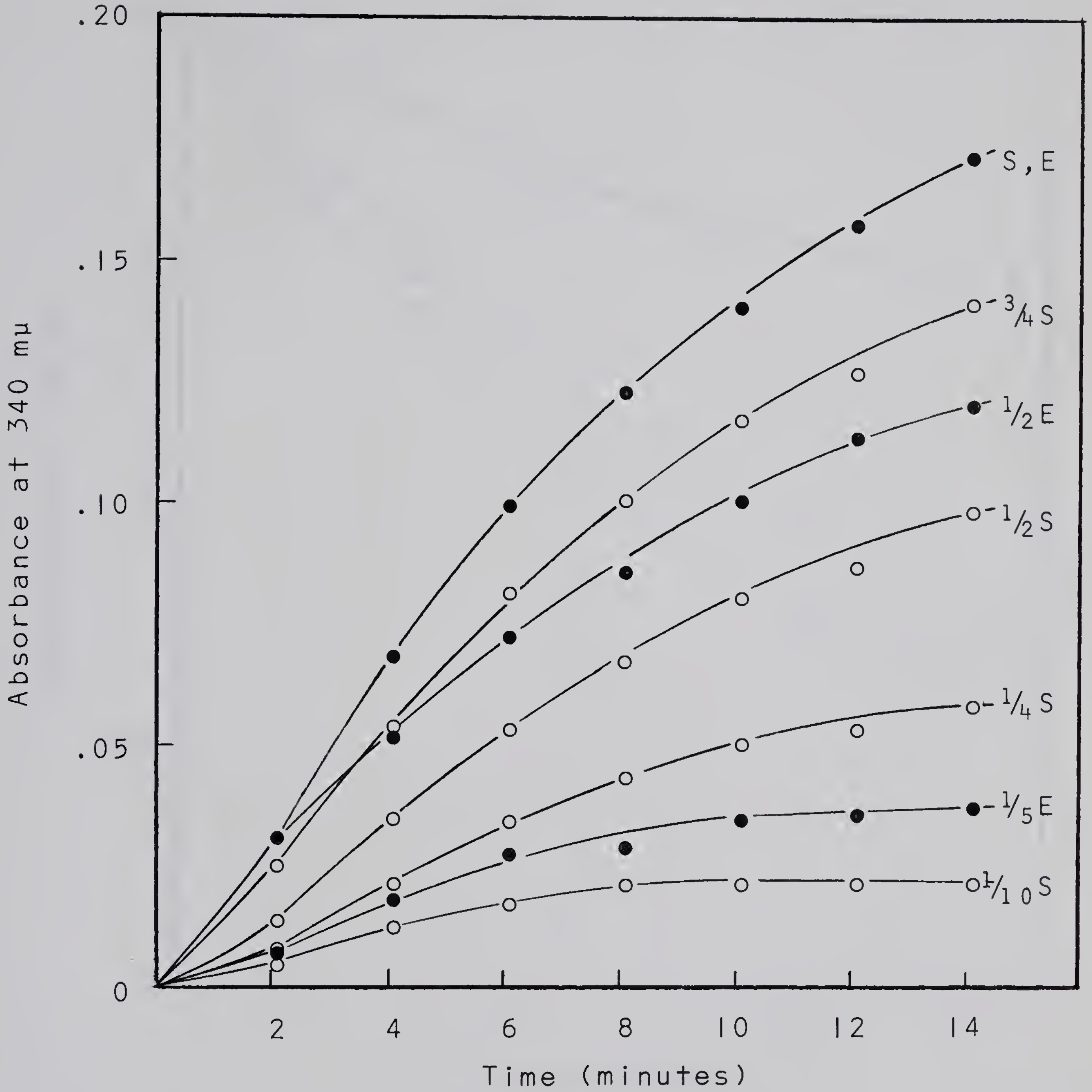


*Sample cells contained 14.8 µmoles of* $Na_2HPO_4$ *buffer (pH 7.4) :*
*with enzyme suspension (2.6 mg protein) +* $Na_3$*Citrate,*
*S 88.4 µmoles*
*½S 44.2 µmoles*
*¼S 22.1 µmoles*
*1/10 S 8.8 µmoles*
*or 88.4 moles of* $Na_3$*Citrate + enzyme suspension,*
*E 2.6 mg protein*
*½E 1.3 mg protein*
*Total volume 3.0 ml.*

[illegible]

FIGURE 15

EFFECTS OF SUBSTRATE AND ENZYME CONCENTRATIONS ON $_{D_S}$-ISOCITRATE:NAD$^+$ OXIDOREDUCTASE ACTIVITY



*Sample cells contained 16.6 μmoles of Tris buffer (pH 7.4) and 1.5 μmoles of NAD$^+$:*
*with enzyme suspension (51.6 mg protein) + Na$_3$Isocitrate,*

| | |
|---|---|
| *S* | *200 μmoles* |
| *3/4S* | *150 μmoles* |
| *1/2S* | *100 μmoles* |
| *1/4S* | *50 μmoles* |
| *1/10S* | *20 μmoles* |

*or 200 μmoles of Na$_3$Isocitrate + enzyme suspension,*

| | |
|---|---|
| *E* | *51.6 mg of protein* |
| *1/2E* | *25.8 mg of protein* |
| *1/5E* | *10.3 mg of protein* |

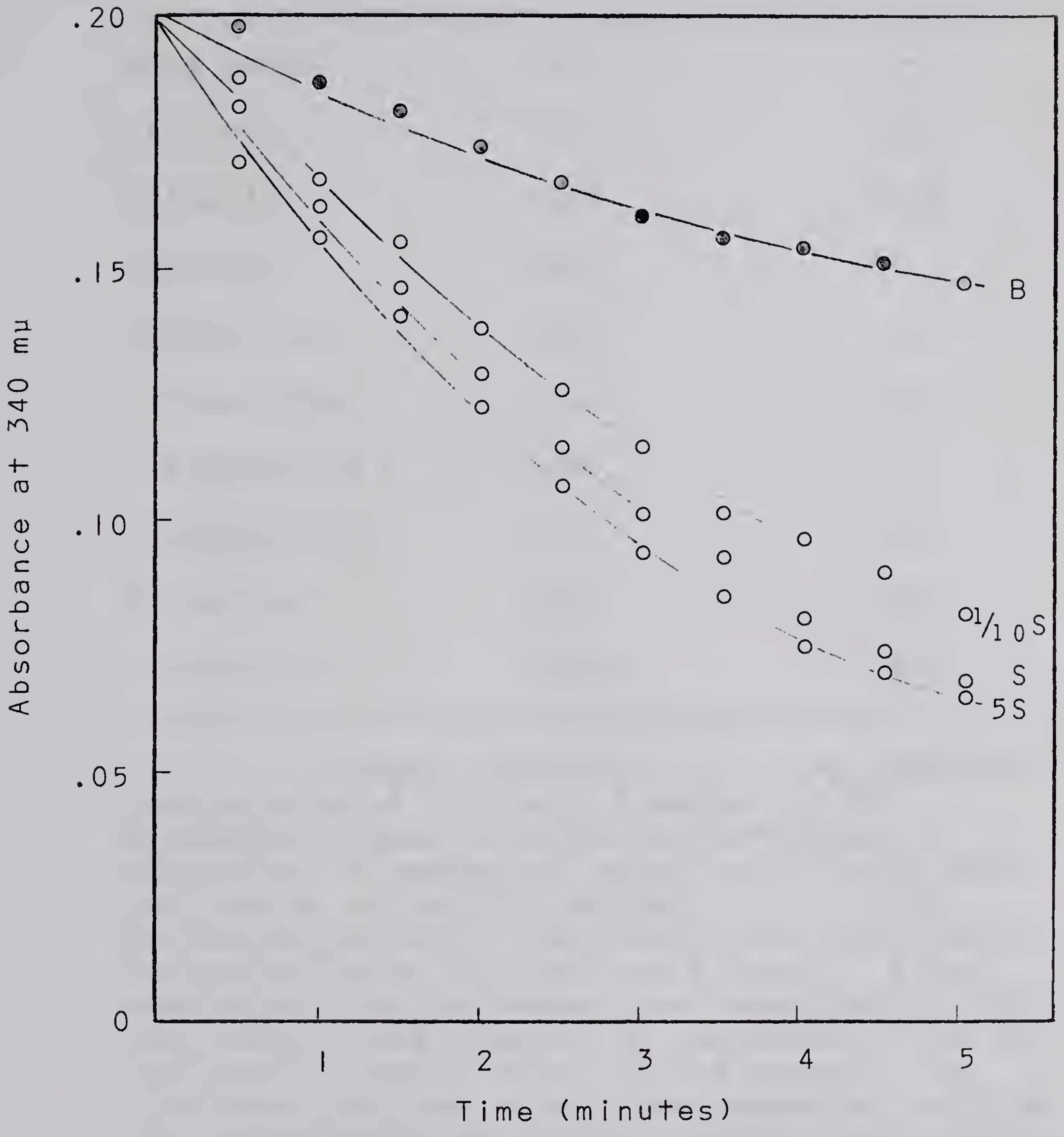
*Total volume 3.0 ml.*

[illegible]

[illegible]

FIGURE 16

EFFECT OF SUBSTRATE CONCENTRATIONS ON L-GLUTAMATE:NAD$^+$ OXIDOREDUCTASE ACTIVITY



*Sample cells contained 150 μmoles of $Na_2HPO_4$ buffer (pH 7.2) and 0.2 μmoles of NADH: with enzyme suspension (20.0 mg protein), 300 μmoles of $NH_4^+$, and K α-ketoglutarate,*

*S 10 μmoles*
*$^1/_{10}$S 1 μmole*
*5S 50 μmoles*

*or 50 μmoles of K α-ketoglutarate + enzyme suspension (20.0 mg protein) with no added $NH_4^+$ (B)*
*Total volume 3.0 ml.*

[illegible]

[illegible]

## TABLE 9

## AMINOTRANSFERASE REACTIONS WITH α-KETOGLUTARATE

| Amino Donor | cpm in Glutamate | % over Control |
|---|---|---|
| None added | 6100 | — |
| Glycine | 7500 | 23.7 |
| D-Alanine | 9500 | 55.6 |
| L-Alanine | 10200 | 67.9 |
| D-Aspartate | 5900 | 0.0 |
| L-Aspartate | 9100 | 49.3 |
| D-Asparagine | 5900 | 0.0 |
| L-Asparagine | 11000 | 80.1 |
| D-Leucine | 7600 | 24.8 |
| L-Leucine | 10700 | 76.0 |

*1.0 ml of enzyme suspension (63.0 mg protein) incubated with 1.0 μc/4.0 μmoles of* $Na_2$ *α-ketoglutarate, 8 μmoles of pyridoxyl-5-phosphate, 2 μmoles of amino acid donor, and 200 μmoles of* $Na_2HPO_4$ *buffer (pH 7.1) for 30 minutes at 25°. The reactions were stopped by the addition of absolute ethanol. After centrifugation to remove the denatured protein, the ethanol was removed by evaporation and the solutes dissolved in distilled water. The glutamate was separated from unreacted acid by ion exchange chromatography using Dowex cation exchange resin in the hydrogen form as described in the Materials and Methods Section.*

## TABLE 10

## SPECIFIC ACTIVITIES AT 25° OF THE ENZYMES STUDIED

| Enzyme | Substrate | Substrate Concentration µmoles/ml | pH | $10^{-3}$ × Specific Activity* |
|---|---|---|---|---|
| Alcohol:NAD$^+$ Oxidoreductase | ethanol | 50.0 | 7.4 | 52.4 |
| Citrate (Isocitrate) Hydro-lyase | citrate | 29.5 | 7.4 | 0.9 |
| D$_S$Isocitrate:NAD$^+$ Oxidoreductase | D,L-isocitrate | 66.7 | 7.4 | 5.6 |
| L-Aspartate:α-Ketoglutarate Aminotransferase | α-ketoglutarate | 1.1 | | |
| | L-aspartate | 0.5 | 7.1 | 7.7 |
| L-Glutamate:NAD$^+$ Oxidoreductase (Deaminating) | | | | |
| dialyzed homogenate† | α-ketoglutarate | 0.3 | | |
| | $NH_4^+$ | 3.3 | 7.2 | 4.3 |
| undialyzed homogenate‡ | α-ketoglutarate | 3.3 | | |
| | $NH_4^+$ | 100.0 | 7.2 | 0.3 |

**units of specific activity - µmoles of substrate transformed/min/mg protein*

†*determined by the conversion of α-ketoglutarate-$C^{14}$ to glutamate-$C^{14}$*

‡*spectrophotometric determination*

# DISCUSSION

The extent of metabolic reactions in any sequences will tend to be affected by the pool sizes of their intermediates. When these pools are extremely large, they exert a great influence on the ultimate incorporation and distribution of radioactive precursors supplied to such systems in minute quantities. In the cotyledons of peas germinated under the conditions of the present experiments, there are three extensive pools associated with the TCA cycle: citrate, glutamate, and homoserine. The pattern of $C^{14}$ incorporation into pea cotyledons can be largely explained in terms of the effect of these pools, coupled with the extensive metabolism of a wide variety of compounds not directly associated with the TCA cycle.

## The Neutral and Lipid Fractions

Whenever two- and three-carbon compounds were supplied to cotyledon slices, significant but generally small amounts of $C^{14}$ were detected in both the neutral and lipid fractions (Tables 1 to 7).

### *Neutral Compounds*

The neutral fraction accumulated $C^{14}$ in two types of compounds, one of which was sugars. Acetate-$C^{14}$ labelled compounds of both types, lactate-$C^{14}$ only sugars, and ethanol-$C^{14}$ and acetaldehyde-$C^{14}$ only a non-sugar substance.

Sugars are produced from PEP by a reversal of glycolysis (Conn and Stumpf 1963). Pyruvate, in equilibrium with lactate, can be converted to PEP by two mechanisms although the actual process in the intact plant is not definitely known (Walker 1962). Lactate was converted principally to sugars in 30 minutes (Table 7). This implies the presence of a glycolytic sequence which is not only active, but readily reversible.

Two-carbon compounds cannot regenerate pyruvate or PEP except by traversing the TCA cycle: oxaloacetate is converted to PEP at the expense of ATP in a readily reversible reaction (Walker 1962). Thus the two-carbon compounds would be expected to label sugars at a much lower rate and this was observed to occur. In fact, ethanol and acetaldehyde produced only traces of labelled sugars after 60 minutes.

Sugar phosphates became extensively labelled from lactate, ethanol, acetaldehyde, and acetate. In fact these compounds became significantly labelled sooner than malate (Figure 11). This anomaly might be explained in terms of a very small metabolic pool of malate which would quickly label oxaloacetate. The decarboxylation step and reverse glycolysis would have to occur very readily to account for such rapid accumulation of $C^{14}$ in hexose phosphates, however. This sequence would not accumulate extensive amounts of $C^{14}$ in hexose phosphates or free sugars due to the active forward glycolytic pathway. This fact was born out by the actual results since the rate of $C^{14}$ accumulation in hexose phosphates

increased rapidly and then levelled off.

Ethanol and acetaldehyde produced a single labelled compound which is probably acetoin (3-hydroxy-2-butanone). There is only indirect evidence for the formation of this compound since the specific test for it devised by Westerfield (1945) gave no reaction. The intermediate in the formation of acetyl CoA, α-hydroxyethyl TPP (Figure 2), can react with either acetaldehyde to produce acetoin directly, or pyruvate to form α-acetolactate which decarboxylated to acetoin (Krampitz *et al* 1962). Enzymes catalyzing both mechanisms have been found in ripening pea seeds by Davies (1964) who showed that excess acetaldehyde stimulated acetoin synthesis. As a consequence, acetaldehyde-1,2-$C^{14}$ would be expected to label acetoin more extensively than ethanol-$C^{14}$. This was observed: the neutral compound from acetaldehyde-$C^{14}$ contained 4 times as much $C^{14}$ as from ethanol-$C^{14}$. Acetate-$C^{14}$, which is not involved with TPP for activation, incorporated only trace amounts of $C^{14}$ into this compound. This evidence suggests that the compound is indeed acetoin and its rapid rate of $C^{14}$ accumulation might indicate such a small pool as to be below the limits of sensitivity of Westerfield's test.

Acetate-$C^{14}$ labelled two additional neutral compounds, which were not identified. Since they became more heavily labelled from acetate-1-$C^{14}$ than from acetate-2-$C^{14}$, it is possible that they arise via $CO_2$ fixation.

### *Lipids*

The lipid fraction of seeds generally consists of triglycerides of various fatty acids (Koller *et al* 1962). Thus $C^{14}$ accumulating either in glycerol or in fatty acids would label the lipid fraction. The early appearance of a labelled lipid fraction suggests that the synthesis does not involve the TCA cycle. Furthermore, lactate-$C^{14}$ labelled this fraction more heavily in 30 minutes than after 120minutes whereas the two-carbon compounds showed a consistently increasing pattern (acetaldehyde-$C^{14}$ excepted). Since lactate-1-$C^{14}$ would not label acetyl CoA, the fatty acid moiety would not accumulate any $C^{14}$; however, as was shown above, glycolysis is readily reversible and this could readily supply glycerol-$C^{14}$ (lactic acid is as soluble in ether as in water but the partition coefficient favors aqueous solution; nevertheless these figures might represent lactate rather than lipid). Two-carbon compounds cannot give rise to glycerol without traversing the TCA cycle so at least initially, their contribution of $C^{14}$ to lipid would be via fatty acid synthesis involving acetyl-$C^{14}$ CoA.

## Reactions Associated with the Tricarboxylic Acid Cycle

By far the greatest portion of the two- and three-carbon compounds supplied in the present experiments entered the acid pools of the TCA cycle, presumably via acetyl CoA. The large pools of citrate and glutamate represented a trap

for $C^{14}$ in all cases and this effect would tend to restrict its recycling. The low rate of $C^{14}O_2$ evolution, which has been interpreted as indicative of sluggish cycle operation, can also be explained by this phenomenon.

Condensation of acetyl-$C^{14}$ CoA and oxaloacetate produces citrate-$C^{14}$ which enters a pool of citrate initially containing little $C^{14}$. This citrate is in equilibrium through the action of citrate (isocitrate) hydro-lyase with isocitrate. Oxidation of isocitrate will cause withdrawl of citrate from its metabolic pool. Since the pool is very large, most of the citrate removed from it as isocitrate will be unlabelled, although the proportion of isocitrate-$C^{14}$ formed will increase as the citrate pool becomes more heavily labelled. In addition, a significant part of the citrate may be present in a storage pool not in ready equilibrium with the metabolic pool as was shown in several plant tissues by MacLennan *et al* (1963). If this situation occurs in the cotyledons of peas, citrate-$C^{14}$ from the metabolic pool would tend to exchange with unlabelled citrate from the storage pool until their specific activities were equal. This would account for a small but continuous removal of $C^{14}$ from equilibrium with the TCA cycle.

Two enzymes compete for α-ketoglutarate, producing succinate by oxidative decarboxylation and glutamate by reductive amination. Although succinate was more heavily labelled than glutamate in the very shortest experiments (utilizing acetate-1-$C^{14}$), glutamate showed a rapid increase in $C^{14}$ accumulation followed by a decline after 30 minutes.

[illegible]

Since the total amount of glutamate in the tissue is increasing at this time (Larson and Beevers 1965), the rise and subsequent fall of $C^{14}$ levels is supported by the findings of MacLennan *et al* (1963) that the glutamate pool may be largely or completely in equilibrium with the cycle. Besides transamination, glutamate can be converted to glutamine or decarboxylated to γ-aminobutyrate. The former reaction is apparently more active since only small amounts of γ-aminobutyrate-$C^{14}$ were detected. It is possible that γ-aminobutyrate might re-enter the TCA cycle by means of a mechanism discussed by Pietruszko and Fowden (1961) found in yeast. In this reaction γ-aminobutyrate undergoes transamination to form succinic semialdehyde which is oxidized to succinate.

The pools of succinate, malate, and fumarate are small, so their dilution effects are probably not significant.

Oxaloacetate appears to serve as an intermediate of several reactions besides condensation with acetyl CoA. These include aspartate production, and hence homoserine; and sugars and glycolytic intermediates by reverse glycolysis. In addition it is the product of $CO_2$ fixation into PEP. In these experiments homoserine and various products of reverse glycolysis became labelled but not asparagine. This is not surprising since the asparagine content is decreasing as germination proceeds (Lawrence and Grant 1963).

By these various reactions additional amounts of $C^{14}$ will be temporarily withdrawn from the cycle. There was also

a continuous increase in the incorporation of $C^{14}$ into other acidic compounds which are neither TCA cycle acids nor hexose phosphates. These compounds were readily displaced from Dowex anion exchange resin (formate) by gradient elution (Figure 4). Glyoxylate-$C^{14}$ and glycollate-$C^{14}$ were the only compounds identified.

Thus one turn of the TCA cycle will label oxaloacetate with only a small proportion of the $C^{14}$ incorporated initially into citrate. Citrate arising from labelled oxaloacetate will mix with the metabolic pool of citrate and therefore be subject to further dilution effects. Theoretically, all of the label incorporated into citrate from acetyl-l-$C^{14}$ CoA will be released as $C^{14}O_2$ on the second turn of the TCA cycle, one-half as a result of the decarboxylation of isocitrate, and one-half from the subsequent decarboxylation of α-ketoglutarate. From methyl-labelled acetyl CoA, both decarboxylations release only one-half of the incorporated $C^{14}$ as $C^{14}O_2$ on the third turn. Each successive turn will further reduce the likelihood of $C^{14}O_2$ release, and this effect would be enhanced by any substantial withdrawl of intermediates from the cycle *e.g.* α-ketoglutarate and oxaloacetate.

Experiments in which isocitrate, α-ketoglutarate, and glutamate were supplied to cotyledon slices supported the conclusions drawn from the data outlined above. Although the principal product of isocitrate metabolism was citrate, the other products of isocitrate utilization were incorporated in

a manner consistent with the sequence under consideration. However, isocitrate was not as effective a precursor of glutamine as were acetate and α-ketoglutarate (Table 8). The incorporation of α-ketoglutarate-5-$C^{14}$ was similar to that observed from the acetate-2-$C^{14}$ experiment. The relatively larger proportions of $C^{14}O_2$ released by isocitrate-5,6-$C^{14}$ and α-ketoglutarate-5-$C^{14}$ do not indicate that extensive cycling of labelled metabolites occurs even after 6 hours. Theoretical considerations show that glutamate produced after one complete turn of the TCA cycle from isocitrate-5,6-$C^{14}$ would have evolved 75% of its label as $C^{14}O_2$ (assuming the distribution of $C^{14}$ between C-5 and C-6 of the supplied isocitrate was 1:1). For glutamate produced from α-ketoglutarate-5-$C^{14}$ after one complete turn of the cycle, 50% of its label would have been so released. Thus a significant proportion of the carbon incorporated from these compounds does not appear to traverse the TCA cycle even once in 6 hours.

Glutamate was much more extensively incorporated into glutamine, γ-aminobutyrate, and the insoluble fraction than the other substrates examined and the amount of label in cycle acids and $CO_2$ was consequently much less. This implies that the rate of oxidative deamination of glutamate was less than that occurring in the other reactions. Furthermore the possibility of an exchange transamination reaction as reported by Hiller and Walker (1961) appears to be slight.

The amount of recycling through the TCA cycle can be conveniently estimated by determining the amount of $C^{14}$ in

C-1 of glutamate. The ultimate equilibrium of $C^{14}$ distribution is attained when the rates of $C^{14}$ entry into the cycle and $C^{14}$ evolution (as $C^{14}O_2$) are equal and the rate at which this equilibrium is approached depends upon the proportion of $C^{14}$ that is recycling. When the glutamate pool has reached this equilibrium with the TCA cycle, C-1 of glutamate should contain one-half as much label as C-5 (*i.e.* 33.3% of the total $C^{14}$ in glutamate) when it is derived from labelled compounds giving rise to acetyl-1-$C^{14}$ CoA. However C-1 never contained more than 5% of the total glutamate $C^{14}$ even after 60 minute incubation periods with such labelled compounds. When the condensation involves acetyl-2-$C^{14}$ CoA, C-1 will contain $^1/_7$ (14.3%) of the total glutamate $C^{14}$ at equilibrium although more recycling would be required to attain this labelling pattern. Labelled substrates of this type incorporated only negligible amounts of $C^{14}$ into C-1 of glutamate. The corresponding figures for isocitrate-5,6-$C^{14}$ and α-ketoglutarate-5-$C^{14}$ (the labelling pattern of which is the same as from acetyl-1-$C^{14}$ CoA) were 4.5% and 10.1% respectively. Glutamate-3,4-$C^{14}$ (corresponding to acetyl-2-$C^{14}$ CoA labelling patterns) incorporated 0.6% of the label into its C-1 position. This evidence lends further support to the postulation that dilution of radioactive metabolites in the citrate and glutamate pools impedes the recycling of such compounds through the TCA cycle.

## Significance of Carbon Dioxide Fixation

An active $CO_2$ fixation system has been found to be present in germinating pea cotyledons (Cossins unpublished data) and the extent to which this system operates probably depends on the amount of carbon withdrawn from the TCA cycle. Davies (1959) has stated that the amount of $CO_2$ fixation should be equal to the amount of acid withdrawn from the cycle if other reactions producing four-carbon acids are not quantitatively significant. L-Malate glyoxylate-lyase (CoA acetylating 4.1.3.2 has been found to possess significant activity in 5-day old cotyledons of germinating peas (Yamamoto and Beevers 1960). To establish whether this activity is present in 3-day old cotyledons, slices were preincubated with glyoxylate before addition of ethanol-$C^{14}$ and acetate-$C^{14}$. In tissues where the glyoxylate cycle is known to occur, pretreatment with glyoxylate enhanced incorporation of both ethanol and acetate (Peterson and Cossins 1965). In the present experiments, however, acetate incorporation was unchanged and that of ethanol was reduced by 31.4%. This inhibitory effect of glyoxylate on ethanol metabolism is apparently not due to the formation of oxalomalate (Ruffo *et al* 1962b). These workers showed accumulation of citrate when pyruvate was supplied to liver and kidney particles in the presence of glyoxylate (Ruffo *et al* 1962a). The present experiments showed no increase in citrate-$C^{14}$, which suggests that the inhibition is not caused by oxalomalate.

The inhibitory effect of glyoxylate could also be explained by formation of a complex with TPP. This reaction has been shown to occur between glyoxylate and TPP (Miller *et al* 1962) and would account for inhibition of ethanol but not acetate incorporation (which oxalomalate inhibition could not).

The conclusion which can be drawn from these experiments is that there is no appreciable glyoxylate cycle activity in the cotyledon tissues since malate glyoxylate-lyase activity has not yet developed. Therefore net synthesis of compounds from the acids of the TCA cycle will require replenishment of those acids, and $CO_2$ fixation remains as the most likely means by which that replenishment could occur.

Fixation of $C^{14}O_2$ appears to account for the incorporation of label into the acids of the TCA cycle from lactate-1-$C^{14}$, since acetyl CoA derived from it would not be labelled. In 120 minutes the C-1 of glutamate contained 74.2% of the total label in this amino acid, which is comparable to the proportions from $C^{14}O_2$ fixation experiments with pea cotyledon slices (Cossins unpublished data). The present experiments cannot be cited as evidence for the extent to which fixation of $CO_2$ is occurring, however. The presence of a strongly alkaline solution in the centre well of the reaction flasks acted as a trap for most of the $CO_2$ released. In addition the amounts of $C^{14}O_2$ evolved were generally very small, so that even moderate amounts of fixation would be undetectable or insignificant when compared with the

large amounts of $C^{14}$ in the supplied substrates.

Several of the sequences associated with the TCA cycle could possibly result in irreversible or negligibly reversible synthetic reactions. The most significant of these products is homoserine. Although each of the reactions in its synthesis is reversible, homoserine is metabolically inactive in pea cotyledon tissues. Even 24 hours after supplying homoserine-$C^{14}$ to cotyledon slices 82% of the label was retained in homoserine (Larson and Beevers 1965). The low rate of accumulation of $C^{14}$ in homoserine may be explained by the dilution effect of the citrate and glutamate pools, in which case net synthesis might be occurring at the expense of TCA cycle acids. Alternatively homoserine may be produced from aspartate resulting from protein hydrolysis. It is probable that aspartate produced by both methods is utilized in homoserine synthesis. The insoluble fraction may represent a similar essentially irreversible synthesis.

The extent of any net synthesis at the expense of TCA cycle acids in plant tissues represents a complex analytical problem much of which remains to be elucidated. Nevertheless, if biosynthesis of cellular components utilizes acids of the TCA cycle without sufficient replenishment of carbon from other sources, there is an active $CO_2$ fixation system which is capable of maintaining the supply of oxaloacetate to sustain cycle operation.

## Comparison of the Metabolism of Ethanol and Acetate

The mechanisms of acetyl CoA generation from ethanol and acetate appear to be quite different (pages 5 and 6) and if both were converted exclusively to acetyl CoA, only the rates of the respective reactions could alter the incorporation sequences.

Ethanol is converted via acetaldehyde to α-hydroxyethyl TPP (Figure 2) and can be subsequently oxidized by lipoate (Breslow 1962) and transferred to CoA. Some of the α-hydroxyethyl TPP is converted to acetoin, however, and this represents a reduction in the amount of ethanol that can give rise to acetyl CoA. Acetate, on the other hand, is transformed to acetyl CoA in a reaction involving hydrolysis of ATP by an enzyme intimately associated with the condensing enzyme (Hiatt 1962). Therefore a greater proportion of supplied acetate is likely to be converted to citrate.

It was noted that ethanol is apparently metabolized more rapidly than acetate (pages 34 and 43). If this represents an actual trend, compounds derived from the TCA cycle, *e.g.* amino acids, should contain a higher proportion of $C^{14}$ from ethanol than from acetate. The rate of accumulation of label in $CO_2$ would likewise depend on the relative amounts of ethanol and acetate traversing the TCA cycle.

In order to test this hypothesis, the amounts of $C^{14}$ incorporated into compounds not derived from acetyl CoA (non-sugar neutral fraction), compounds converted to acetyl

CoA but not traversing the TCA cycle (lipid fraction), and compounds derived from the cycle (amino acids, amides, and $CO_2$) were compared to the amounts of $C^{14}$ incorporated into the organic acid fraction. The results of the comparisons are shown in Table 11. It can be seen that a significant proportion of the ethanol was removed (as acetoin) from participation in the cycle. Ethanol nevertheless labelled the amino acids and amides more heavily than acetate but not lipids. Apparently ethanol and acetate are equally effective precursors of fatty acids. The $CO_2$ data are not consistent but discrepancies might be explained by the relatively large errors in detecting very low·levels of $C^{14}$ in $BaCO_3$.

These factors were further examined by comparing the effects of preincubation of cotyledon slices with acetate or ethanol upon addition of micromolar amounts of ethanol-$C^{14}$ and acetate-$C^{14}$ respectively (Table 12). Ethanol had no effect on acetate-$C^{14}$ incorporation while acetate reduced ethanol-$C^{14}$ incorporation and increased the proportion of acetoin-$C^{14}$. The labelling ratios were also determined (Table 13). The proportions of incorporation into lipids and amino acids were not appreciably altered by any combination of metabolites. A comparison cannot be made between $C^{14}O_2$ released from ethanol-2-$C^{14}$and acetate-1-$C^{14}$ since one was methyl-labelled and the other carboxyl-labelled. However preincubation did not affect the evolution of $C^{14}O_2$ from either substrate compared with the amount of $C^{14}$ in organic acids. The most

## TABLE II

## COMPARISON OF LABELLING RATIOS OF ETHANOL-$C^{14}$ AND ACETATE-$C^{14}$

| | 5 min | 15 min | 30 min | 60 min |
|---|---|---|---|---|
| cpm in Lipid Fraction/cpm in Organic Acid Fraction | | | | |
| Ethanol-1-$C^{14}$ | 0.06 | 0.09 | 0.11 | 0.14 |
| Acetate-1-$C^{14}$ | 0.05 | 0.09 | 0.13 | 0.15 |
| Ethanol-2-$C^{14}$ | 0.08 | 0.10 | 0.13 | 0.17 |
| Acetate-2-$C^{14}$ | 0.05 | 0.07 | 0.10 | 0.08 |
| cpm in Non-Sugar Neutral Fraction/cpm in Organic Acid Fraction | | | | |
| Ethanol-1-$C^{14}$ | 0.12 | 0.15 | 0.11 | 0.11 |
| Acetate-1-$C^{14}$ | 0.06 | 0.02 | 0.02 | 0.01 |
| Ethanol-2-$C^{14}$ | 0.20 | 0.12 | 0.11 | 0.11 |
| Acetate-2-$C^{14}$ | 0.03 | 0.01 | 0.02 | <0.01 |
| cpm in Amino Acid and Amide Fractions/cpm in Organic Acid | | | | |
| Ethanol-1-$C^{14}$ | 0.66 | 1.32 | 1.75 | 1.58 |
| Acetate-1-$C^{14}$ | 0.59 | 1.09 | 1.26 | 1.37 |
| Ethanol-2-$C^{14}$ | 0.53 | 0.93 | 1.30 | 1.22 |
| Acetate-2-$C^{14}$ | 0.32 | 0.74 | 1.48 | 1.42 |
| cpm in $CO_2$ Fraction/cpm in Organic Acid Fraction | | | | |
| Ethanol-1-$C^{14}$ | 0.01 | 0.02 | 0.06 | 0.16 |
| Acetate-1-$C^{14}$ | 0.02 | 0.05 | 0.11 | 0.19 |
| Ethanol-2-$C^{14}$ | <0.01 | <0.01 | <0.01 | 0.01 |
| Acetate-2-$C^{14}$ | 0.01 | 0.01 | 0.01 | 0.01 |

*The figures for the cpm are taken from Tables 1, 2, 4, & 5*

## TABLE 12

### INCORPORATION OF ETHANOL-2-$C^{14}$ AND ACETATE-1-$C^{14}$ IN THE PRESENCE OF ACETATE AND ETHANOL

| Fraction | Ethanol-2-$C^{14}$ |  | Acetate + Ethanol-2-$C^{14}$ |  | Acetate-1-$C^{14}$ |  | Ethanol + Acetate-1-$C^{14}$ |  |
|---|---|---|---|---|---|---|---|---|
|  | cpm | % of total | cpm | % of total | cpm | % of total | cpm | % of total |
| Lipid | 9600 | *4.1* | 2100 | *2.8* | 34200 | *4.6* | 40200 | *5.1* |
| Neutral |  |  |  |  |  |  |  |  |
| sugars | not detected |  | not detected |  | trace | *0.0* | 11200 | *1.4* |
| others | 20400 | *8.7* | 21200 | *28.1* | 16000 | *2.1* | 5700 | *0.7* |
| Acidic Amino Acid |  |  |  |  |  |  |  |  |
| glutamate | 54500 | *23.3* | 14400 | *19.1* | 154300 | *20.6* | 175700 | *22.3* |
| aspartate | 8400 | *3.6* | 900 | *1.2* | 23000 | *3.1* | 21500 | *2.7* |
| others | trace | *0.0* | trace | *0.0* | 8000 | *1.1* | 9700 | *1.3* |
| Amide | 9000 | *3.8* | 3100 | *4.1* | 46700 | *6.2* | 38000 | *4.8* |
| Neutral and Basic Amino Acid | 5300 | *2.3* | 1500 | *2.0* | 14400 | *1.9* | 22800 | *2.9* |
| Organic Acid | 105900 | *45.2* | 26600 | *35.2* | 329800 | *44.1* | 337200 | *42.8* |
| Carbon Dioxide | 1500 | *0.6* | 500 | *0.6* | 83000 | *11.1* | 87700 | *11.1* |
| Insoluble | 19800 | *8.4* | 5200 | *6.9* | 38800 | *5.2* | 38400 | *4.9* |
| Total $C^{14}$ Incorporated | 234400 |  | 75500 |  | 748200 |  | 788100 |  |

*0.5 g of slices preincubated with 50 µmoles of $NaH_2PO_4$ (pH 5.5) and 10 µmoles of Na Acetate or Ethanol as indicated at 30° for 30 minutes before addition of 5 µc/µmole of Ethanol-2-$C^{14}$ or 5 µc/µmole of Na Acetate-1-$C^{14}$ for an additional 30 minutes at 30°. Total volume 0.7 ml.*

TABLE 13

LABELLING RATIOS OF THE ETHANOL/ACETATE COMPETITION EXPERIMENT

| | Lipid | Non-Sugar Neutral | Amino Acid & Amide | $CO_2$ |
|---|---|---|---|---|
| Ethanol-2-$C^{14}$ | 0.09 | 0.19 | 0.73 | 0.01 |
| Acetate + Ethanol-2-$C^{14}$ | 0.08 | 0.80 | 0.75 | 0.02 |
| Acetate-1-$C^{14}$ | 0.10 | 0.05 | 0.75 | 0.25 |
| Ethanol + Acetate-1-$C^{14}$ | 0.12 | 0.02 | 0.79 | 0.28 |

*The figures for the cpm are taken from Table 12. The ratio is a comparison between the fraction indicated and the organic acid fraction.*

significant effect of acetate on incorporation of ethanol-2-$C^{14}$ was the extensive accumulation of acetoin-$C^{14}$ almost certainly as a result of inhibition of acetyl CoA formation. Since the distribution of $C^{14}$ within the cycle and in lipids was virtually the same, only the fate of ethanol and acetate during the sequence of formation of acetyl CoA must result in different metabolic products. Preincubation with acetate reduced ethanol-2-$C^{14}$ metabolism by 67.8% and resulted in accumulation of acetoin-$C^{14}$. Therefore acetate must act as a non-competitive inhibitor of ethanol conversion to acetyl CoA.

This interpretation is also consistent with the different rates of $CO_2$ evolution from ethanol and acetate in rat tissue using labelling ratios (Russell and Van Bruggen 1964). It can account for similar results in plant tissues (Castelfranco *et al* 1963) using an extension of a premise actually stated, but not employed, in the paper, *i.e.* that differences in metabolic fates could result if all or part of the ethanol were funnelled into a sequence not involving free acetate.

# CONCLUSIONS

These studies have indicated the presence in pea cotyledons of an exceedingly active tricarboxylic acid cycle which is of primary importance in the metabolic reactions of germination. Labelled metabolites are rapidly incorporated into the acids of the TCA cycle, accumulating extensively in the large pools of glutamate and citrate. Glutamate and oxaloacetate are in equilibrium with various sequences that tend to withdraw $C^{14}$ from the cycle, which accounts for the significant labelling in a large number of other compounds. The combined effect of these trends impedes the recycling of the supplied $C^{14}$ through the TCA cycle so that little labelled $CO_2$ is released.

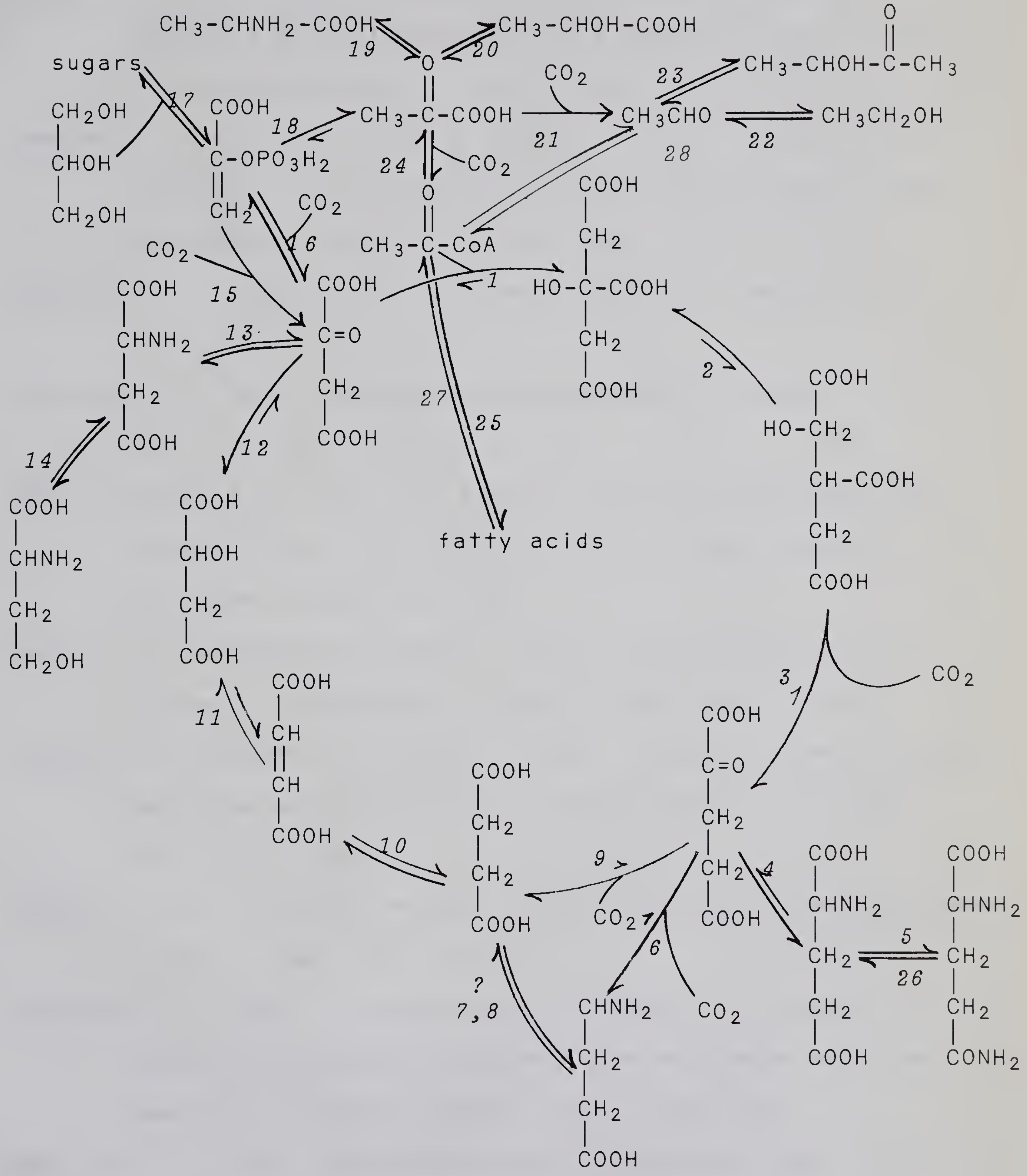
The presence of an active $CO_2$ fixation system was demonstrated although its significance in germination reactions could not be estimated from these experiments.

The metabolic sequences found to be operating in this tissue are shown in Figure 17. The large number of reactions in equilibrium is responsible for the complex patterns of $C^{14}$ incorporation and also for the low rate at which it is recycled through the TCA cycle.

1 citrate oxaloacetate-lyase (CoA acetylating) 4.1.3.7
2 citrate (isocitrate) hydro-lyase 4.2.1.3
3 threo-D -isocitrate:NAD$^+$ oxidoreductase (decarboxylating) 1.1.1.41
4 L-glutamate:NAD$^+$ oxidoreductase (deaminating) 1.4.1.2
5 L-glutamate:ammonia ligase (ADP) 6.3.1.2
6 L-glutamate 1-carboxy-lyase 4.1.1.15
7 L-γ-aminobutyrate:α-ketoacid aminotransferase 2.6.1.-
8 succinate semialdehyde:NAD$^+$ oxidoreductase 1.2.1.3
9 α-ketoglutarate:lipoate oxidoreductase (acceptor acylating) 1.2.4.2
succinyl CoA:dihydrolipoate S-succinyltransferase 2.3.1.-
NADH:lipoamide oxidoreductase 1.6.4.3
succinate:CoA ligase (ADP) 6.2.1.5
10 succinate:FAD oxidoreductase 1.3.99.1
11 L-malate hydro-lyase 4.2.1.2
12 L-malate:NAD$^+$ oxidoreductase 1.1.1.37
13 L-aspartate:α-ketoglutarate aminotransferase 2.6.1.1
14 ATP:L-aspartate 4-phosphotransferase 2.7.2.4
L-aspartate β-semialdehyde:NADP$^+$ oxidoreductase (phosphorylating) 1.2.1.11
L-homoserine:NAD$^+$ oxidoreductase 1.1.1.3
15 orthophosphate:oxaloacetate carboxy-lyase (phosphorylating) 4.1.1.31
16 ATP:oxaloacetate carboxy-lyase (transphosphorylating) 4.1.1.32
17 glycolytic enzymes
18 ATP:pyruvate phosphotransferase 2.7.1.40
19 L-alanine:α-ketoacid aminotransferase 2.6.1.12
20 L-lactate:NAD$^+$ oxidoreductase 1.1.1.27
21 pyruvate carboxy-lyase 4.1.1.1
22 alcohol:NAD$^+$ oxidoreductase 1.1.1.1
23 acetoin acetaldehyde-lyase 4.1.2.-
24 pyruvate:lipoate oxidoreductase (acceptor acylating) 1.2.4.1
acetyl CoA:dihydrolipoate S-acetyltransferase 2.3.1.12
NADH:lipoamide oxidoreductase 1.6.4.3
25 enzymes catalyzing fatty acid synthesis (malonyl CoA)
26 L-glutamine amidohydrolase 3.5.1.2
27 β-oxidation enzymes
28 aldehyde:NAD$^+$ oxidoreductase (acylating CoA) 1.2.1.10

FIGURE 17

METABOLIC REACTIONS OF THE COTYLEDONS OF GERMINATING PEAS ASSOCIATED WITH THE TRICARBOXYLIC ACID CYCLE



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